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# Microbiology and the Limits to Life in Deep Salts



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# Lay summary

Despite some salt deposits being over 250 million years old, they host a range of salt-adapted microbes, some of which are hypothesised to be thousands, even millions of years old. How exactly these communities survive in these environments is still unknown. Interest has spiked recently in these environments due to the discovery of similar salt deposits on Mars. Whilst surface conditions on Mars are very hostile to life, the subsurface may provide a safe haven for any extant life on the planet. On Earth, subsurface salt deposits are remarkably common and can be found underlying a significant portion of the planet's landmass.

The importance of deep subsurface salt deposits to both terrestrial and extraterrestrial science means that our current understanding of life in these environments needs to be improved. This thesis addresses this by significantly advancing our understanding of how the deep biosphere is shaped by salts. It achieves this by sampling waters and salt rocks from an active mine to examine the impact brine and rock salt geochemistry has had on the native microbial communities. The work shows how the remains of a 250-million-year-old sea can still control the habitability of modern deep subsurface waters, sometimes even rendering them completely uninhabitable to all known life.

Cutting edge sequencing technologies are used to gain insights into the composition and behaviour of the communities living in these environments. Their remarkable similarity to highly salty environments on the Earth's surface demonstrates that salt can almost exclusively shape microbial communities, regardless of environmental setting.

How microbial communities survive in these environments was also investigated. Ancient sources of carbon were examined to see if the organisms from the salt deposit could use them for sustenance. As well as showing this to be possible, it was found that certain salt rocks greatly increased the growth of the deep subsurface organisms by changing the water chemistry around them. Gases that could provide energy and carbon sources also were found trapped in salt rocks. Potential positive detections of Mars relevant metabolisms were also made, adding to the relevance of the brines as Mars analogue environments. Overall this work greatly enhances the established knowledge base of these fascinating environments.

# Abstract

Deep subsurface evaporites are common terrestrial deep subsurface environments found globally. These deposits are known to host communities of halophilic organisms, some of which have been suggested to be millions of years old. The discovery of evaporite minerals on Mars has led to these environments becoming of interest to astrobiology, particularly because the subsurface of Mars represents the best chance of finding more clement conditions conducive to life. Despite this interest, deep subsurface evaporites remain poorly understood and we have little insight into how different salts shape the Earth's biosphere, much of which is underground. This thesis addresses several knowledge gaps present in the literature by sampling a selection of brine seeps and rock salt samples taken from Boulby Potash Mine, UK. The origin and evolution of the brines is determined with geochemical techniques, showing the majority to have been sourced from an aquifer above where they were intersected in the mine. These brines appear to have taken a variety of pathways through the subsurface leading to the presence of a range of different ions dissolved within them. The majority are Na/Cl dominated, whilst one is K/Cl dominated. One brine appears to have a different origin and probably interacted with dolomite becoming very concentrated in Mg. This variety in brine origins and migration pathways has impacted the habitability of the brines. Physicochemical measurements for chaotropicity, water activity and ionic strength, combined with culturing experiments suggest brines from the Sherwood Sandstone were habitable, but the brine from a distinct unknown source was uninhabitable. DNA was successfully extracted from three of the habitable brines and their metagenomes sequenced. These revealed communities largely functionally and phylogenetically similar to surface near saturation brines, indicating that the structure of the communities present in saturated Na/Cl brines are controlled almost exclusively by these ions rather than any other environmental difference between the surface and subsurface. Organisms were also taken from these brines and culturing experiments carried out to determine if any carbon sources were present in ancient salt that might promote growth in the absence of other carbon sources. Controls showed that the geochemical changes to the growth media induced by solving the salts, particularly sylvinite, were responsible for the increases in growth observed, indicating certain salt minerals effectively fertilise the growth of halophiles. Culturing on hydrocarbon seeps collected in the mine suggested they may provide a carbon source periodically to some organisms within the deposit. Work was done to show the presence of dissimilatory sulphate and iron reducing halophiles. Overall this significantly advances our understanding of how

salts shape the Earth's biosphere, particularly its deep subsurface component, and what functional capabilities life has to persist in these environments. This work provides a new window on the potential habitability of deep subsurface extraterrestrial environments and how we might go about investigating these environments for habitable conditions.

## **Declaration**

This thesis has been solely composed by the candidate. Unless clearly stated, this work is the candidate's own. Where results have been obtained through collaborations with other researchers, their contributions are made clear in the text as appropriate.

This work has not been submitted for any other degree or professional qualification.

Candidate's signature

Date.....12/01/2017.....

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# Chapter 1: Introduction

Evaporite deposits are the remains of water bodies that become concentrated in dissolved ions and eventually dried up, depositing these ions as layers of salt. Examples of modern evaporite deposits include the salt flats of Death Valley in the USA, or the Dead Sea. These processes have been common over geological time, meaning buried ancient evaporites make up a significant part of the terrestrial deep subsurface.

The discovery of evaporites on Mars led to new interest in these deposits as Mars analogue sites. The inhospitable Martian surface conditions dictate that its subsurface environments are the best places to search for both modern extant life and the remains of ancient biology. Known to contain halophiles, some of which have been suggested to be millions of years old, deep subsurface environments represent a terrestrial example of highly specialised microbial communities seemingly isolated from the surface for long periods of geological time. Since the Martian surface was only thought to be habitable billions of years ago, any deep subsurface communities present would need to be able to survive for exceptionally long periods of time cut off from the surface. For these reasons, terrestrial deep subsurface evaporites have become of increasing astrobiological interest.

Despite this interest, relatively little is known about the microbial communities present within these deposits and their associated brines. These knowledge gaps include determining how these organisms obtain energy and carbon, and the impact of brine origin and composition on community structure and habitability. Understanding more about these environments will help improve our knowledge of a globally distributed deep subsurface terrestrial environment that no doubt plays important roles in deep subsurface processes. In addition, it will help assess the habitability of analogous extraterrestrial environments, allowing future missions the greatest chance of successfully selecting a habitable environment to explore.

## 1.1 Thesis scope, objectives and hypotheses

The core objective of this thesis was to develop an understanding of how salts shape the deep subsurface biosphere. To fill the variety of knowledge gaps outlined above and detailed in Chapter 2, this thesis uses materials collected from Boulby Potash Mine to answer several questions addressing the work's core objective. It achieves this through a diverse set of studies involving geochemistry, sequencing and culturing techniques. Effectively, three top level questions are addressed:

**Q1.** How do different salts determine the habitability of the deep subsurface?

Hypothesis: Interactions between waters and the different salts in the Zechstien evaporite sequence will lead to the formation of brines containing a range of ions at different concentrations. This will result in physicochemical changes in some extreme brines that push them into uninhabitable regimes.

**Q2.** What influences do brine environment and migration routes have on the phylogenetic and functional profile of microbial communities?

Hypothesis: Variation in dissolved ionic species will be influenced by brine origin and migration routes through the subsurface, resulting in changes to microbial community composition.

**Q3.** How do organisms in deep subsurface evaporite brines acquire carbon and energy for growth?

Hypothesis: Lack of sunlight, low oxygen and slow environmental turnover will drive microbial community functional adaption, including developing methods to exploit novel recalcitrant carbon sources and carrying out anaerobic processes to acquire energy.

## 1.2 Key concepts

Habitability is an important concept discussed in several sections of this thesis. A habitable environment is defined here as an environment capable of supporting the activity (cell maintenance and/or reproduction) of one or more known organisms (Cockell et al. 2016), meaning an uninhabitable environment is unable to support any known organism. The term “known organism” makes the definition practical as it is not possible to test the growth of every potential form of life in the universe.

This binary definition of habitability means that a 110 °C deep sea hydrothermal vent (an environment that might be described as extreme or hostile) is defined as habitable despite only being able to support the most extreme thermophiles. Long term cell dormancy adds additional complexity. An organism may be able to survive for long periods of time in a state of dormancy within an uninhabitable environment, only to return to activity when placed in a habitable environment. If this organism can maintain a sustainable state of dormancy in that environment, i.e. can repair molecular damage at the rate it accumulates it, then the environment would be considered habitable. It would be considered uninhabitable if the organism could not maintain dormancy and slowly degraded.

A significant nuance to recognise within this definition is that a habitable environment does not have to be inhabited. These “uninhabited habitats” are rare on Earth due to the ubiquity of biology, but may be present on other planets with more transient habitable environments. Such planets may have lacked an origin of life, or input from panspermia, and consequently when habitable environments appeared no life was present to exploit them.

The concept of habitability is complex and multifaceted, and the discussion above outlines the definition used in this thesis. However, for a comprehensive review of the many permutations of habitability, see Cockell et al. (2016).

## 1.3 Thesis outline

A range of different interlinked studies are documented in this thesis. The methods and techniques used are detailed in each individual chapter where appropriate. Chapter 2 provides an overview of the existing literature pertaining to terrestrial deep subsurface evaporites, focusing on their microbiology. It also covers the literature related to habitability in brine environments and the presence of evaporites on Mars.

Chapter 3 provides the geochemical context for the remaining chapters by examining the ionic and mineralogical composition of the brines and salt rocks collected from Boulby Potash Mine, UK. Through this, the origin and evolution of the brines is inferred. This chapter also tests for the presence of gases trapped in salt rocks. Chapter 4 employs a range of techniques to investigate the habitability of the brines collected by examining their physicochemical properties and carrying out enrichment experiments. Chapter 5 then interrogates the metagenomics profile of the microbial communities present in a number of brines using next generation sequencing methods, and compares them to surface hypersaline environments. Chapter 6 attempts to determine the carbon usage capabilities of the brine microbial communities, with particular focus on carbon present in the ancient salt rock. Finally, Chapter 7 details enrichment experiments that test for the presence of organisms capable of dissimilatory iron and sulphate reduction in the brines. Chapter 8 summarises and synthesises the overall findings and recommends research directions for future workers.

## Chapter 2: Background

This thesis explores the geochemistry and microbiology of a deep subsurface evaporite deposit in order to answer a number of questions related to how organisms survive in these environments, the links between brine origin and habitability and the role of dissolved ions in shaping brine community structure. The presence of evaporites on Mars and their assumed extension into its deep subsurface has led to increasing interest in these widespread, but often poorly explored deep subsurface terrestrial environments. It is worth noting that the term “deep subsurface” is often undefined, but is used here loosely to mean below ~ 50 m in depth. The following literature review examines the microbiology and habitability of terrestrial hypersaline environments, with focus on deep subsurface evaporites and then introduces some of the evidence for evaporites on Mars. It aims to highlight the types of organisms found in these environments, the adaptations they display and their relationship with surface environments. Although the term “hypersaline environment” typically refers to NaCl rich brines, in reality high concentrations of a number of other salts can be found in various terrestrial brines, including in deep subsurface evaporites. These have the potential to elucidate a better understanding of terrestrial and extraterrestrial fluid habitability. Therefore, the physicochemical limits they impose on habitability are also discussed here.

### 2.1 Terrestrial deep subsurface evaporites

Life on Earth was once believed to exist as a thin blanket covering the planet’s surface. The truth, however, is that life on Earth exists wherever space, water, energy and carbon sources permit. It is now estimated that between 1% and 30% (by carbon weight) of the biosphere may reside in the deep subsurface, although estimates have moved towards the lower end of this range recently (Parkes et al. 1994, Whitman et al. 1998, Lipp et al. 2008, Hinrichs and Inagaki 2012, Kallmeyer et al. 2012). Even at the lower end of this estimate, this is still a substantial portion of the terrestrial biosphere that resides in the deep subsurface.

Deep subsurface evaporites are an important and common deep subsurface environment on Earth. For example, they are thought to underlie 35-40 % of the US (Davies and LeGrand

1972) with some sequences being hundreds of meters thick. This makes them a significant bio-space in the terrestrial subsurface and of great ecological and biogeochemical significance.

### **2.1.1 Microbiology of deep subsurface evaporite deposits**

Despite their importance globally, deep subsurface evaporite deposits remain understudied environments. The following sections examine some of the work carried out on organisms from a variety of deep subsurface evaporite materials, including rock salt, brines and sediments, taken from boreholes and salt mines. It includes some discussion about the types of organisms present, how these organisms are adapted to this environment and how they compare to surface hypersaline environments. The potential antiquity of these halophiles is also examined.

#### **2.1.1.1 Microbial fauna in deep subsurface evaporites**

Like in their surface counterparts, all three domains of life have been identified in deep subsurface evaporites. These were sourced from a mixture of materials, including organisms trapped in halite crystals and a variety of brine and salt mine materials such as mine soil, air samples and effervescences. In some of the salt mine studies, organisms may have been transported into the mine by anthropogenic activity, or may be native. Microbiology results for these materials are still discussed here, since it is still plausible that they originate from the deep subsurface evaporite deposit and omitting them completely would also mean a substantial aspect of the literature would be unaddressed. Where there is uncertainty about this aspect, it is highlighted in the text.

A number of novel strains have been isolated and characterised from deep subsurface evaporites. The following tables display a range of these novel strains and details some of their basic characteristics.

	<i>Halococcus salifodinae</i>	<i>Halobacterium noricense</i>	<i>Haloferax chudinovii</i>	<i>Halococcus dombrowskii</i>	<i>Halosimplex carlsbadense</i>	<i>Halobacterium salinarum</i>	<i>Halobacterium hubelense</i>
Depth (m)	n/a	350	250	650	n/a	186	2000
Specific environment	Halite rock	Halite rock	Sylvinite rock	Halite rock	Halite crystal	Halite crystal	Halite
Domain	Archaea	Archaea	Archaea	Archaea	Archaea	Archaea	Archaea
Colony colour	Pink	Light red	Pink to red	Pink to red	Pink to red		
Temp. range (°C)	28-50		23-51	28-50	22-50	<40	22-50
Temp. optimum (°C)	40	37-45		37-40	37-40		45
NaCl range (%)		>12.5	7-27	>15			
NaCl optimum (%)	20-25	15-17.5	15-18	20-25	25	20.5	14.6
pH range	6.8 - 9.5	5.2-7	6.8-7	5.8-8	7-8		
pH optimum			5.5-8			7.5	
Nitrate reduced to nitrite	+	-	-	+	-	-	+ (genes present)
Oxygen requirements	Aerobic (unspecified)	+		+		+	+
	Aerobic Obligate		+		+		
	Aerobic Facultative		+				
References	Denner et al. 1994	Gruber et al. 2004	Saralov et al. 2013	Stan-Lotter et al. 2002	Vreeland et al. 2002a	Mormile et al. 2003	Jaakkola et al. 2016b

**Table 2.1.** A range of archaeal isolates from deep subsurface evaporite deposits worldwide.

The majority have come from salt crystals in Permian aged deposits, apart from *Halobacterium salinarum*. All of them are also aerobic and display high salt requirements for growth. The majority have come from halite, except for *Haloferax chudinovii*, which was isolated from sylvinite.

	<i>Salinicoccus kunningensis</i>	<i>Salinicoccus albus</i>	<i>Halomonas salifodinae</i>	<i>Fodiniibacter luteus</i>	<i>Fodiniibius salinus</i>	<i>Gracilimonas mengyeensis</i>	<i>Thermobifida halotolerans</i>	<i>Zhihengliuella saisuensis</i>	<i>Alifodiniibius roseus</i>	<i>Alifodiniibius sediminis</i>
Depth (m)	200	200	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Specific environment	Brine	Brine	Saline soil in a mine	Wall of a salt mine	Sediment from salt mine	Sediment from salt mine	Salt mine (unspecific)	Brine	Sediment from salt mine	Sediment from salt mine
Colony color	Yellow	White	Brown-yellow	Orange-yellow	Pink	Pale red	n/a	Light yellow	Rose red	Salmon pink
Temp. range (°C)	4-45	5-40	4-48	15-37	25-45	20-37	20-50	10-40	20-42	25-45
Temp. optimum (°C)	37	25	30	28	37	28		25-30	28	28
NaCl range (%)	0.5-25	1-30	0.5-20	2.5	4-23	2-15	<10	0.5-20	4-20	4-16
NaCl optimum (%)	8-10	10	3		10-15	5-9		5-10	6-10	6-10
pH range	6-10	6-10	6-9		6-9	6.5-8	6-9	6.5-10.5	6.5-8	6.5-8.5
pH optimum	8	8.5	7	6.5-7.5	7.5-8	7.5		8.5	7	7
Nitrate reduced to nitrite	+	+	-	+	+	+	+	-	-	+
Oxygen requirements	Aerobic (unspecified)		+	+			+	+		
	Aerobic Obligate	+	+		+					+
	Aerobic Facultative									
	Anaerobic Facultative					+			+	
Reference	Chen et al. 2007	Chen et al. 2009	Wang et al. 2008	Wang et al. 2009	Wang et al. 2012	Wang et al. 2013a	Yang et al. 2008	Chen et al. 2010	Wang et al. 2013b	Wang et al. 2013b

**Table 2.2.** A range of bacteria isolated from deep subsurface evaporite deposits worldwide. Unlike the archaea, none were isolated from halite crystals. Instead they came from a range of materials in salt mines. The exact sampling context in these studies were often poorly described, leading to questions about their representativeness of the deep subsurface evaporite. The depth of these deposits was also generally unavailable.



	<i>Phialosimplex salinarum</i>	<i>Selenaion koniopes</i>
Age of deposit	Permian	Permian
Depth (m)	n/a	n/a
Specific environment	Brine	Brine
Domain	Eukaryotes	Eukaryotes
Temp. range (°C)	4-35	20-40
NaCl range (%)	4-25	1.5-15
NaCl optimum (%)	n/a	~5
Aerobic (unspecified)	+	+
Reference	Greiner et al. 2014	Park et al. 2012

**Table 2.3.** Characteristics of two Eukaryotes isolated from deep subsurface evaporite deposits. Both are aerobic and were isolated from brine samples. *Selenaion koniopes* is an Amoeba and *Phialosimplex salinarum* a filamentous fungus.

Other examples not listed above include, *Marinococcus tarijensis* (Balderrama-Subieta et al. 2013), *Halolamina salina* and *Halolamina salifodinae* (Zhang et al. 2013a), *Halopelagius longus* (Zhang et al. 2013b), *Halopiger salifodinae* (Zhang et al. 2013c), *Amycolatopsis cihanbeyliensis* (Tatar et al. 2013), *Halomonas qiaohouensis* (Wang et al. 2014), *Bacillus salitolerans* (Zhang et al. 2015a) and *Aquibacillus salifodinae* (Zhang et al. 2015b) *Halobaculum roseum* (Chen et al. 2016).

It is clear that a range of organisms exist in deep subsurface evaporites, in both man-made mines and trapped in rock salt. The salt requirements of the isolates vary, with the archaea having optimum NaCl concentrations from 14.6-25 % salinity and the bacteria generally more moderate 3-15 %. The majority grow in a roughly neutral pH range, with optimum

temperatures ranging from 25 to 45 °C. The majority are aerobic organisms and many can reduce nitrate to nitrite.

The levels of contamination involved in several of these studies is unclear, owing to a lack of clarity on the sampling context. This makes it unclear how many of the isolated organisms truly came from deep subsurface evaporite deposits and invites criticism about the prevalence of aerobic organisms. Generally, the archaea isolated from rock salt had a degree of surface decontamination performed due to the authors' desire to examine the antiquity of the trapped organisms (e.g. Denner et al. 1994, Gruber et al. 2004, Stan-Lotter et al. 2002, Vreeland et al. 2002, Mormile et al. 2003, Jaakkola et al. 2016b). However, many of the studies involved in isolating the bacteria in Table 2.2 do not detail the context of the samples collected (e.g. Chen et al. 2010, Wang et al. 2012). For example, isolating organisms from materials like sediment from salt mines may include material transported in from the surface. The most likely source for the majority of the halophilic organisms is probably still the evaporite deposit, but contamination is a concern and worth considering when interpreting the provenance of these organisms.

Culture independent techniques have also revealed a similar spread of organisms, with many uncharacterised organisms identified (Fendrihan et al. 2006). Cloned 16S rRNA sequences have shown the presence of organisms closely related to members of the Halobacteriaceae family, many of which remain uncultured and unclassified (Radax et al. 2001, Fish et al. 2002, Fendrihan et al. 2006, Sankaranarayanan et al. 2014, Jaakkola et al. 2016a). Lipid profiles examined by Norton et al. (1993) from two salt mines found they were related to a range of Halobacteriaceae such as *Halococcus* spp. *Halobacterium salinarium*, *Halobacterium saccharovorum* (now *Halorubrum saccharovorum*, McGenity and Grant 1995) and *Haloarcula* spp. as well as a number of unidentified lipids. Xiao et al. 2013, found a range of bacterial and archaeal 16S rRNA sequences present in salt mine soils, the closest relatives being organisms such as *Halanaerobium fermentans*, *Halomonas ventosae*, *Halovibrio denitrificans*, *Halorubrum litoreum*, *Haloarcula japonica*, *Halomicrobium mukohataei* and *Haloarcula tradensis*. Others have found a range of bacterial DNA in a number of different places in a salt mine, including *Bacillus megaterium*, *Virgibacillus halodenitrificans* and *Aquifex pyrophilus* amongst others, although the authors were predominantly looking at salt quality (Carpa et al. 2014) and these organisms could be related to contamination.

Some modern deep subsurface sediment sequences have been drilled into and had cores extracted. In the halite and gypsum sediments, found below 200 m under the sea floor of the Dead Sea, Halobacteria were found to dominate the communities present in gypsum layers, with bacterial sequences only making up a small portion of the communities. The salt saturated aragonite/mud laminae, however, showed more diversity containing a range of genera, including *Methanomicrobia*, *Thermococci*, *Halobacteria* and *Archaeoglobi* (Thomas et al. 2014). In both layers, fermentation appeared to be an important metabolism and in the aragonite/mud laminae, methanogenesis also appeared to be significant. This work combined with later studies on a different core showed that the microbial community composition in the subsurface sediments of the Dead Sea does not vary significantly (Thomas 2015).

#### **2.1.1.2 Comparison to surface hypersaline environments**

The types of organisms living within surface hypersaline environments is often dependant on salinity. Shifts in community structure will occur as water evaporates and the brine becomes more concentrated. In less saline regions of areas such as the Great Salt Lake, US, cyanobacteria thrive (Oren 2014). As salinity increases, *Dunaliella*, a salt adapted micro-algae, outcompete the cyanobacteria and become the primary producers. *Dunaliella* can survive from around 3 % (w/v) to saturation (Chen et al. 2009), but optimal growth tends to be around 6-15 % NaCl (Brock 1975, Oren 2014). Evaporation ponds may display colourful microbial mats on the bottom of the pond, made up of anoxygenic phototrophs and cyanobacteria, such as those at Guerrero Negro (salinity around 9 %) (Spear et al. 2003, Oren 2015a). As NaCl reaches saturation levels, surface environments become dominated by Halobacteriaceae (Oren 2002) and sometimes other specially adapted organisms such as *Salinibacter ruber* (Antón et al. 2002). An exception of this was found in muddy salt saturated hypersaline anoxic sediments at the bottom of a crystalliser pond, where sulphate-reducing bacteria dominated (López-López et al. 2010). At its most concentrated, the Dead Sea can reach salinities approaching 35 %, with high concentrations of  $Mg^{2+}$  and  $Ca^{2+}$  present. This prevents even the most extreme halophiles from forming any dense microbial communities (see Section 2.1.1.5 for more detail on ion interactions and habitability). However, during dilution events such as intense rainfall, blooms of Halobacteriaceae can be observed (Oren 2010, Oren 2015a).

Most halophiles examined in deep subsurface evaporites do appear relatively similar to those found on the surface. For example, genes found in the genomes of *Halobacterium hubeiense*

and *Halococcus salifodinae*, organisms isolated from rock salt, were almost all homologous to characterised surface haloarcheal genes (Jaakkola et al. 2016a, 2016b). Larger clone libraries show the bacterial and archaeal communities present in the soil of salt mines to be similar to those found in surface hypersaline environments close to saturation, with Halobacteriales dominating the archaeal sequences and Bacteroidetes, Proteobacteria, and Firmicutes the bacterial sequences (Xiao et al. 2013). Generally, the organisms isolated from rock salt samples do not show dramatic divergence from surface halophiles (Graur and Pupko 2001, Nickle et al. 2002, Hazen and Roedder 2001), although some, such as *Halosimplex carlsbadense*, show a degree of divergence (Vreeland et al. 2002a). However, the analysis of deep subsurface evaporite materials, including brines, rock salt and fluid inclusions, has still yielded numerous new species and genera as shown in Section 2.2.1.1, although there is a lack of studies utilising next generation sequencing techniques and comparing them holistically to surface environments.

### 2.1.1.3 Adaptations to the hypersaline deep subsurface

The presence of halophiles in deep subsurface evaporites naturally leads to the question of how such organisms survive in these environments. Whilst the answer to this is not entirely clear currently, the halophiles do have a number of adaptations that may help them deal with the extremes presented by deep subsurface environments. A stressor all organisms living in hypersaline environments must deal with is salinity.

Increasing concentrations of NaCl require a variety of adaption techniques utilised by different groups of organisms. At NaCl concentrations approaching saturation, organisms tend to incorporate inorganic ions, typically  $K^+$  and  $Cl^-$ , into their cytoplasm to maintain osmotic pressure. This method is commonly referred to as the “salt-in” strategy. The family Halobacteriaceae are most commonly associated with this method of salt adaptation. However, there are a range of other organisms which also employ this strategy, including Halanaerobiales, a fermentative anaerobic bacterium, and two aerobic bacteria, *Halorhodospira* (Deole et al. 2013) and perhaps most famously, *Salinibacter ruber* (Antón et al. 2002, Makhdoumi-Kakhki et al. 2012). A combination of convergent evolution and potentially some lateral gene transfer appear to be responsible for these similar adaptations methods appearing in these distantly related organisms (Mongodin et al. 2005).

Organisms with the salt-in adaptation typically display a proteome containing higher abundances of acidic amino acids such as glutamic and aspartic acid (Kennedy et al. 2001).

This allows the physical structure and therefore mechanical functionality of the proteins to be maintained through hydration shells when ions are competing for water molecules (Lanyi 1974, Karan et al. 2012).

K<sup>+</sup> may not be the only salt-in osmolyte used by halophiles. Fagerbakke et al. (1999) found evidence that certain marine bacteria contain cytoplasmic Mg<sup>2+</sup> concentrations an order of magnitude higher than the surroundings environment. This led them to suggest that this organism is using Mg<sup>2+</sup> as an osmoprotectant.

In contrast to the salt-in strategy, the salt-out strategy attempts to exclude inorganic ions from the interior of the cell by synthesising or procuring organic solutes (Oren 2011a), these normally consist of low molecular weight, uncharged or zwitterionic molecules such as amino acids, betaines and the sugar trehalose (Grant 2004). Since these molecules are uncharged or zwitterionic, they do not alter the hydration shells surrounding proteins and therefore the proteome of these organisms does not need to be specifically adapted. For this reason, organisms using this strategy tend to inhabit a greater range of salinities than those employing the salt-in method (Ventosa et al. 1998), but are out competed at the highest salinities when producing/scavenging extremely high quantities of compatible solutes becomes less energetically favourable.

These methods of haloadaptation directly impact the types of metabolisms utilised by the organisms in hypersaline environments. In the salt-out method, constantly pumping in or producing compatible solutes is energetically expensive, particularly synthesising these solutes. Similarly, energy is required to sustain sharp K<sup>+</sup>/Na<sup>+</sup> boundaries at the cell membrane when an organism is using the salt-in method (Oren 1999, 2011b). Consequently, only a small group of metabolisms can be used at the highest salt concentrations, including aerobic respiration, photosynthesis, denitrification and fermentation (see Oren 2011b for more detail).

Despite these often being considered as separate adaption strategies, work done on *Halorhodospira* by Deole et al. (2013) suggests that when the organism is presented with an environment low in KCl, but high in NaCl, its cytoplasmic concentrations of K reduce and internal glycine concentrations increase. In this way, the organism is capable of both the “salt-in” and “salt-out” strategies. Its ability to move to glycine production also allowed the organism to function over a broad range of salinities, indicating the possession of an acidic proteome does not necessarily preclude successful functioning at lower salinities.

In addition to the stressors imposed by a concentrated NaCl brine, the deep subsurface brings additional challenges such as low nutrient content. Halophiles are well adapted to dealing with oligotrophic environments. Changes in cell morphology have been examined as a method employed by certain organisms to attempt to increase surface-to-volume ratio in order to improve nutrient uptake. One example of a halophile achieving this can be seen in *Haloquadratum walsbyi*, which appears to flatten itself when presented with a nutrient poor environment to increase surface-to-volume ratio and maximise phototrophic growth (Bolhuis 2005, Stan-Lotter and Fendrihan 2015). Another method demonstrated in *Haloferax volcanii* is the ability of some halophiles to grow on DNA sourced from the surrounding environment. This organism was also able to utilise its own polypoidal DNA as a phosphate source (Zerulla et al. 2014). These capabilities could potentially help halophiles deal with the slow environmental turnover and low nutrient levels in the deep subsurface by providing these organisms with a storage mechanism for certain nutrients and a nutrient source in the form of extracellular DNA brought in from adjacent geological environments.

The deep subsurface is also typically low in oxygen content and the majority of Halobacteriaceae are aerobic chemoheterotrophs. Similarly, low oxygen conditions are fairly common in concentrated surface brines. However, it is not entirely clear how these organisms deal with this low oxygen content (Oren 2013). Hypotheses generally revolve around a number of alternative energy production methods. Denitrification, which produces close to the same amount of ATP as aerobic respiration, has been observed right up to NaCl saturation (Oren 2011b) and has been demonstrated in several Halobacteriaceae such as *Haloferax denitrificans*, *Haloferax mediterranei*, *Halogeometricum borinquense*, *Haloarcula marismortui* and *Haloarcula vallismortis* (Hochstein and Tomlinson 1985, Mancinelli and Hochstein 1986, Tomlinson et al. 1986, Montalvo-Rodriguez et al. 1998, Oren 2013). Fermentation offers another potential metabolism, particularly the fermentation of arginine (Oren 2013). *Halobacterium salinarum*, for example, uses this method to deal with oxygen limited conditions (Baliga et al. 2002, Wimmer et al. 2008). The reduction of both dimethyl sulfoxide (DMSO) and trimethylamine N-oxide (TMAO), have also both been shown to support the growth of Halobacteriaceae under anaerobic conditions (Oren and Trüper 1990). Dissimilatory fumarate reduction has also been observed in Halobacteriaceae such as *Haloferax denitrificans* (Oren 1991, 2013).

Despite these alternatives, nitrate, arginine, DMSO and TMAO are not often found at substantial concentrations in hypersaline environments, which has led to questions about the true importance of these metabolisms (Oren 2013). Another method some Halobacteriaceae

are capable of is increasing cell buoyancy through gas vesicle production in order to move into regions of the water column higher in oxygen content. However, this adaption has only been observed in a small number of species such as *Halogeometricum borinquense* (Montalvo-Rodriguez et al. 1998, Oren 2013) and is of little use in the deep subsurface. Similarly, phototrophic processes are important for some Halobacteriaceae such as *Halobacterium salinarum* (previously known as *Halobacterium halobium*), which uses a bacteriorhodopsin as a light driven proton pump (Lozier et al. 1975), but are again useless in the deep subsurface. Generally, little is truly known about how this highly significant group of organisms deals with low oxygen conditions.

Other groups of halophiles can use a number of strategies to deal with low oxygen conditions. These include dissimilatory sulphate reducers such as *Desulfovibrio halophilus*, *Desulfovibrio marinus* and *Desulfohalobium retbaense* (Caumette et al. 1991, Thabet et al. 2007, Ollivier et al. 1991), fermentative organisms such as *Haloanaerobium praevalens* and *Haloplasma contractile* (although the latter can use denitrification as well) (Zeikus et al. 1983, Antunes et al. 2008a) and nitrate and selenite reducing organisms such as *Selenihalanaerobacter shriftii* (Blum et al. 2001) (see Oren 2016 for more examples). However, as mentioned, Halobacteriaceae dominate at high NaCl concentrations and any moderate halophiles such as those listed above are normally outcompeted and make up only small portions of the community if present.

#### **2.1.1.4 Long term survival?**

Ever since Reiser and Tasch (1960) and Dombrowski (1963) discovered the first viable organisms in ancient salt rocks, the question of the age of organisms trapped in these environments has been debated. Intuition might suggest that highly salt adapted organisms living hundreds of meters below the surface have most likely been isolated for considerable periods of geological time. The migratory subsurface pathways through which they would be required to travel from a hypersaline surface environment, such as a saltern, to a deep subsurface evaporite would frequently involve passage through fresher environments where most extreme halophiles would perish, particularly given the majority of species of Halobacteriaceae lyse at salt concentrations below 100 g/l (Oren 2011a). It is therefore perhaps not unreasonable to hypothesise that the halophilic communities present in ancient deep subsurface evaporites are remnants of an original microbial populations that once inhabited the ancient water bodies that deposited the salt.

The discovery of viable halophilic archaea isolated from a fluid inclusion within a seemingly unaltered and uncontaminated salt crystal lead to suggestions that halophiles had survived for 250 million years trapped inside these fluid inclusions (Vreeland et al. 2000). Similar work has also claimed to identify archaea within a 121-112-million-year-old (MYA) salt crystal (Vreeland et al. 2007) and in crystals ranging from 23, 121 and 419 million years old (Park et al. 2009). The Park et al. (2009) study found differing microbial populations in the inclusions, with the 23 MYA crystals containing predominantly *Haloarcula* and *Halorubrum*, and the 121 and 419 MYA crystals containing *Halobacterium* and some unclassified groups, leading them to suggest that these inclusions represent ancient and more modern populations of organisms. Similar Halobacteriaceae (*Halococcus salifodinae*) have been found in different Permian evaporite deposits around the world, which has led to suggestions that these organisms represent the remains of populations dating back to that period (Stan-Lotter et al. 1999). Much younger deposits have also yielded viable organisms from inclusions, such as in 34,000 year old salt crystals (Schubert et al. 2010a), where only 0.6 % of crystals tested contained viable halophilic archaea. This is hypothesised to be possible by the entrapment of starved or dead *Dunaliella* which provided the halophilic archaea with organic compounds like glycerol for maintenance (Schubert et al. 2009, Schubert et al. 2010b).

The results of these studies still prove controversial, particularly those involving organisms purported to be millions of years old. Criticisms have often focused on the apparent lack of difference between organisms claimed to be ancient, and those still living in modern surface environments, as well as calling into question the antiquity of the fluid inclusions based on their chemical composition (e.g. Graur and Pupko 2001, Hazen and Roedder 2001, Nickle et al. 2002). Even with entrapment of organic nutrients and the various adaptations halophiles have to slow environmental turnover and background radiation (Stan-Lotter and Fendrihan 2015), it is unclear at present if these organisms can survive for time periods numbering in the millions of years, and if so, how exactly this is achieved. If it is possible, then it potentially has implications for environments like Mars. Although Martian evaporites are much older, it would indicate halophilic organisms are incredibly well suited to the long periods of dormancy that would no doubt be required for survival in the Martian deep subsurface (see Section 2.3.1).



### 2.1.1.5 Habitability in brines

As is clear from the discussion above, NaCl saturation restricts life in these environments to specially adapted organisms. In this way, Na/Cl does not alone render an environment uninhabitable. In contrast, high concentrations of other salts do appear capable of making solutions functionally uninhabitable by altering various aqueous physicochemical properties (discussed below). For example, the behaviour of hydration shells around a chloride ion in a solution changes in a solution with  $Mg^{2+}$  instead of  $Na^+$  (Ohtaki and Radnai 1993). The types of changes can be so severe that they prevent all known life from proliferating in a liquid given sufficient concentrations (e.g. Stevenson et al. 2015a). Such interactions are extremely complex and are only just beginning to be understood, particularly in natural brines containing numerous dissolved ions.

Brines can be found in the terrestrial deep subsurface that are not dominated by NaCl. Shield or cratonic brines, often found in large Precambrian provinces, such as those in Canada and northern Europe, frequently contain highly concentrated brines. These are often Ca/Cl rich, with concentrations up to saturation levels. Their origins are somewhat enigmatic (Warren 2016), with explanations including buried and concentrated ancient marine waters (Bottomley 1994, 1999) and water-rock in situ interactions (e.g. Frape et al. 1984). Although ubiquitous across Precambrian provinces, a small amount of work has focused on the microbiology of these types of fluids (e.g. Onstott et al. 2009; Itävaara et al. 2011, Doig 1995), but particularly lacking is the study of the more concentrated Ca/Cl brines. Examples of brines also dominated by similar ions have been documented in deep subsurface evaporite deposits, such as the main field site for this thesis (see Bottrell et al. 1996 and Section 2.3), but the microbial communities are unstudied.

It is believed a combination of factors work to determine the habitability of brine environments, depending on the ions present. Studies have tended to focus on top-down approaches; monitoring how changes in cell density, RNA or other proxies for habitability shift with ionic composition, and then attempting to identify and separate out some consistent physicochemical parameters that correspond to an apparent change from habitable to uninhabitable. Through this work, a range of different physicochemical stressors have been highlighted from a number of authors which can lead to solutions becoming uninhabitable.

Water activity, which describes the availability of water in a solution (Grant 2004), has long been identified as a key limiter on habitability, particularly in food science (e.g. Scott 1957, Slade et al. 1991). A solution containing high quantities of dissolved ionic compounds will have a portion of the available water molecules trapped in hydration shells around charged solutes. If the concentration of these solutes is too high, water molecules become unavailable for organisms in the solution. Life as we know it requires water to function, (Chaplin 2006) meaning if the water activity is too low, the solution becomes uninhabitable.

Water activity is measured as the partial vapour pressure of water in a substance, divided by the partial vapour pressure of pure water at the same temperature. Water activity is determined as the ratio between the relative humidity of air above pure water and the solution in question in a sealed container at the same temperature. Pure water has a water activity of 1, a saturated solution of NaCl has a water activity of 0.755. At present, the lowest water activity where biological functioning (the germination of a fungus, *Xeromyces bisporus*) has been recorded at is 0.605, although cell division has not been observed at this limit (Pitt and Christian 1968, Williams and Hallsworth 2009). The current lowest water activity for cell division is at 0.611 (Stevenson et al. 2015a).

Water activity is often considered a fundamentally important limiter in solution habitability. It is often invoked when discussing the habitability of Martian brines (e.g. Tosca et al. 2008) and is considered a constraint on the habitability in a number of terrestrial environments such as Don Juan Pond in Antarctica and deep-sea Lakes Discovery and Kryos in the Mediterranean (Hallsworth et al. 2007, Samarkin et al. 2010, Yakimov et al. 2015, Fox-Powell et al. 2016).

Chaotropicity and kosmotropicity describe the effect particular dissolved solutes have on macromolecular systems. Chaotropicity refers to the destabilising or disordering of these macromolecular systems. Kosmotropicity provides the counterpart, describing a stabilising effect. Whilst these terms have been previously used to describe the precipitation or solubilisation of proteins by ions (salting-in/out) in the Hofmeister series (e.g. Sachs and Woolf 2003), efforts have been made to employ the terms to describe the overall impact of a bulk solution on macromolecules. In this latter definition, which is used throughout this work, neither term attempts to describe the underlying causes of this reordering effect, only the end result of the complex solvent interactions underlying it (Ball and Hallsworth 2015). Consequently, chao/kosmotropicity when considered under this definition, can be seen as black box concepts (see Section 4.1.5 for chao/kosmotropicity measurement techniques).

Like water activity, it is presumed that a threshold exists beyond which concentrations of chaotropic substances make replication and survival of biological organisms impossible. This has been demonstrated in the interface region of Lake Discovery, when other stressors such as water activity are benign (Hallsworth et al. 2007). There is no evidence currently that an upper limit to life exists for kosmotropicity. It is worth noting that this ordering or disordering of macromolecules may not always be detrimental to biology. For example, in a cold environment, the ordering effect of kosmotropic substances may be problematic due to the already limited solute interactions. However, a chaotropic substance would promote these interactions and therefore could lower the minimum temperature in which life can exist (Chin et al. 2010).

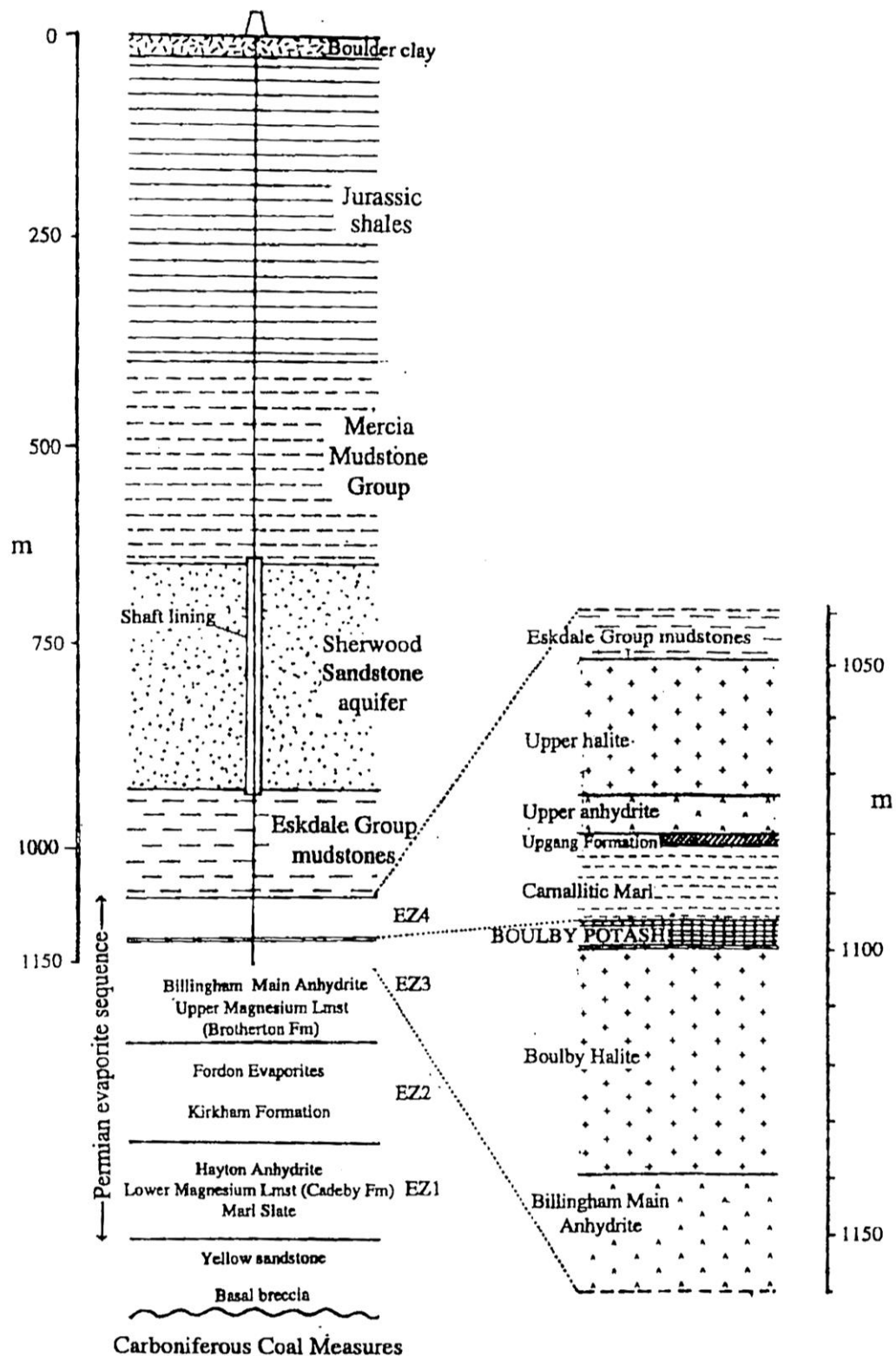
Ionic strength is a unit of charge density. Despite being identified as able to disrupt macromolecules (e.g. Baldwin, 1996; Kunz et al., 2004), charge density had not been considered as a limiting factor in the habitability of brine environments until recently. Investigations into modelled (Tosca et al. 2011) and then synthetically produced Martian brines by Fox-Powell et al. (2016) showed that in combination with other stressors, brines with a high ionic strength could render solutions uninhabitable. Work on this aspect of habitability is currently limited to this one study. Broader ranges of inoculums and sampling natural high ionic strength brine environments will better define the limits for life in high ionic strength brines.

A combination of these factors will be important to consider when studying concentrated brines in deep subsurface environments with atypical (not Na/Cl dominated) ionic compositions. The primary field site for this thesis, Boulby Potash Mine, is dominated by NaCl brines, but samples of brines rich in Mg and Ca have been intercepted at the mine.

## **2.2 Boulby Potash Mine**

Boulby Potash Mine provides direct access to a deep subsurface evaporite deposit and is the main field site for this thesis. Boulby is an active salt mine located in Cleveland, northeast England. The mine primarily extracts potash in the form of sylvinite, but also other salt minerals for a number of purposes. It is the UK's only working potash mine and one of the deepest mines in Europe, reaching 1.3 km at the bottom of the main shaft. Geologically, the mine is located in the Cleveland Basin at the western margin of the North Sea Basin, in a

Zechstein evaporite sequence (Talbot et al. 1982). The Zechstein evaporite sequences are the remains of a large epeiric sea, the deposits of which are spread from the east coast of the UK to Eastern Europe. The sequence at Boulby (see Figure 2.1) is composed of repeating cycles of dolomite, anhydrite, halite, carnallite and shale (Davidson 2009). The potash layer exploited by the mine is in the Upper Permian Teeside Group (English Zechstien cycle EZ3). Below this layer, is the Boulby Halite, in which most of the mine roadways are built. Figure 2.1, shows the local stratigraphic column at Boulby.



**Figure 2.1.** Boulby stratigraphic column from Bottrell et al. (1996). The main roadways of the mine are built in the more mechanically competent Boulby Halite layer. The Boulby Potash horizon above this is the main economic interest in the mine. These two horizons were where most of the sampling in this thesis carried out was achieved.

Very little work investigated the microbial communities in Boulby Mine. Norton et al. (1993) sampled halite and sylvinite rock salt samples, and several brine pools and efflorescences. Boulby samples yielded halobacteria enrichments with viable counts ranging from  $2 \times 10^2$  to  $5 \times 10^6$  c.f.u. ml<sup>-1</sup>. Only one positive enrichment was observed when using a total of 500 g of surface sterilised rock salt fragments (weighing 2-5 g each) to start liquid culture enrichments. Colonies were selected randomly from plate spreads and polar lipid analysis carried out on them. Profiles of the Boulby samples matched those of organisms *Halobacterium saccharovorum* (now *Halorubrum saccharovorum*, McGenity and Grant 1995), *Haloarcula* spp. and *Halobacterium Salinarium* as well as a number of unidentified glycolipids. They concluded that although the salt has been extracted from the mine since 1973, contamination from the mine air was unlikely to be the source of halophiles, suggesting instead they were sourced from the Permian salt deposit.

Similar brine seeps to those sampled by Norton et al. (1993) were examined using hydrogen and oxygen isotopes as well as geochemical assays by Bottrell et al. (1996). They found the majority are sourced from the Sherwood Sandstone aquifer above the mine, or from a mixture of waters of crystallisation released from gypsum dehydration to anhydrite and trapped interstitial sea water below the mine, and other groundwater from meteoric sources. Other work at Boulby has focused on local structural geology (Talbot et al. 1982) and the relationship between faulting and fluid movement (Davison 2009). More information about the specific sites sampled as part of this thesis can be found in Chapter 3.

Most of the work on the Sherwood Sandstone aquifer waters has taken place in the NW of England, away from the Boulby site. According to radiometric age studies, here the aquifer waters are often stratified and range from Holocene to late Pleistocene in age (Andrews and Lee 1979; Edmunds and Smedley 2000). These findings match the more limited work examining Sherwood Sandstone water age at Boulby, where the Sherwood Sandstone waters that enter the evaporite sequence have an isotopic signature that falls in a zone between Holocene and Pleistocene groundwaters (Bottrell et al. 1996). This suggests some connection to modern meteoritic waters in the Sherwood Sandstone at Boulby, with significant turnover occurring in glacial periods.

## 2.3 Water and Martian habitability

Martian geological history is typically split into three major epochs, the Noachian (~4.1 to 3.7 Gyr), Hesperian (3.7 to 3.0 Gyr) and Amazonian (3.0 to present) (Carr and Head 2010). The surface of modern Mars is largely inhospitable. The planet's atmosphere is thin and provides little protection from ultraviolet (UV) radiation (Cockell et al. 2000). Additionally, Mars lacks a magnetic field capable of deflecting significant amounts of ionising radiation (Dartnell et al. 2007). Both radiation sources are thought to cause significant damage to organic compounds. This, combined with the low average temperatures and pressures (below 273 K and 6.1 mbar) (Malin and Edgett 2000) means that microbial activity is unlikely to be present on the surface of the planet. However, a range of geomorphological and mineralogical surface features indicate that in its ancient past, the planet was very different.

The surface of Mars is covered in the remains of ancient channel systems and mineralogical evidence for water. These include outflow channels; huge flood channel systems with high width to depth ratios that typically start in the southern highlands and cut down through the Martian dichotomy into the northern lowlands (Jakosky and Phillips 2001, Burr 2005, Harrison and Grimm 2008). The outflow channels date predominantly from the Hesperian period. More complex dendritic networks also exist with drainage densities comparable to terrestrial examples (Hynek and Phillips 2003, Craddock et al. 2003). It is thought these channels may have been formed by precipitation and run-off processes, indicating an active hydrological cycle on the planet (Mangold et al. 2004) during the Noachian (Carr and Head 2010).

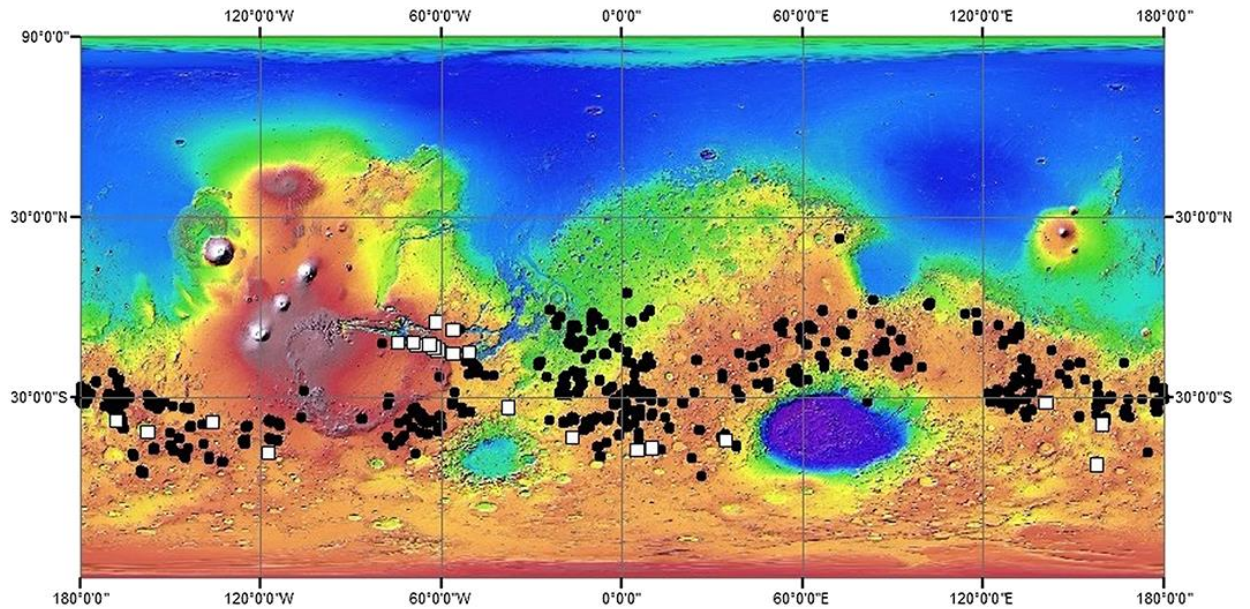
Mineralogical evidence for liquid water is also abundant. Whilst the surface of Mars is covered in unaltered minerals such as pyroxene, olivine and plagioclase feldspar (Christensen et al. 2008), there are some regions that show distinct evidence of alteration by aqueous processes. Phyllosilicates are found across the Martian surface and date to the Noachian period. These minerals form when primary volcanic minerals interact with water (Poulet et al. 2005). Some ferric oxide minerals such as haematite when found within sedimentary rocks (Christensen et al. 2000, 2001b) or as small spherules (Christensen et al. 2004), also provide evidence for hydrological activity on the planet. Finally, evaporite minerals record a number of different periods of ancient and modern aqueous activity, discussed in more detailed below.

### 2.3.1 Evaporites and brines on Mars

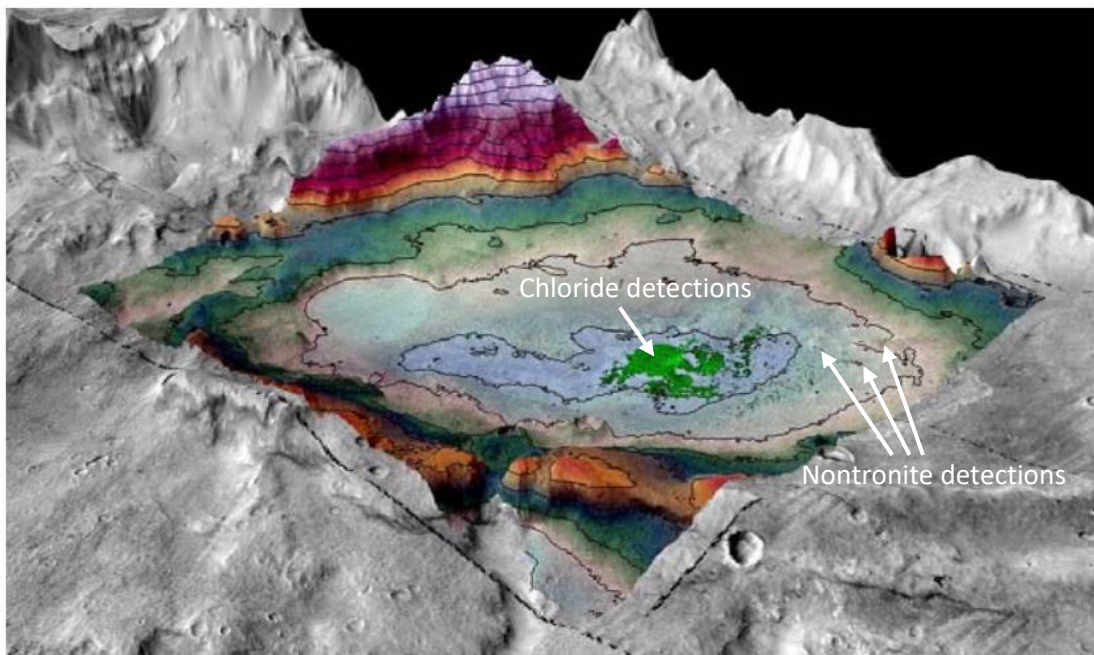
Evaporite minerals make up a substantial part of Martian stratigraphy. Towards the end of the Noachian, throughout the Hesperian and into the early Amazonian (Carr and Head 2010), extensive volcanism led to the presence of sulphate-rich fluids that deposited sulphate rich horizons. An example of this can be seen in the Burns Formation at Meridiani Planum (Hynek 2004, McLennan et al. 2005) which is composed largely of sulphate salts, or the sulphate horizons in the upper and lower members of Gale crater (Milliken et al. 2010). Similarly, in the dust disturbed globally around Mars, sulphates make up the dominant salt component (Yen et al. 2005). The exact mechanism behind the formation of these sulphate deposits is still being debated (Flahaut et al. 2014), and a number of mechanisms could be responsible for different formations, including evaporative processes from sulphate-rich brines (McLennan et al. 2005, Bibring et al. 2006).

Early meteorite evidence also suggested that chloride minerals were present on Mars (Bridges and Grady 1999; 2000). Chloride salt deposits have now been identified in many hundreds of locations over the surface of Mars (Hynek et al. 2015). Osterloo et al. (2008; 2010) identified approximately 640 separate locations displaying chloride salts using images from the Mars Odyssey Thermal Emission Imaging System (THEMIS) (see Figure 2.2). These were all small, localised deposits. Despite being located in depressions, some appeared older than the surrounding materials, suggesting they are exposures of an underlying deposit. Their wide spread distribution over the southern highlands also indicated they are more laterally extensive than is conveyed by their discontinuous appearance, which could be related to their susceptibility to weathering (Osterloo et al. 2010). The formational history of these chloride deposits is still under investigation, but some potential mechanisms include playa lakes fed by run-off or groundwater, efflorescence through atmospheric interactions or hydrothermal brines (Osterloo et al. 2010). It is likely that multiple mechanisms are responsible for the different deposits. Detailed investigations into one of these deposits by Hynek et al. (2015), lead to it being interpreted as an impact crater that was infilled by a lake which later evaporated, became concentrated and deposited chloride salts (see Figure 2.3).





**Figure 2.2.** Locations of chloride minerals (enlarged black boxes) identified on the surface of Mars, plotted on a MOLA cylindrical projection elevation map from 180°W to 180°E and 60°N to 60°S (Osterloo et al. 2010). The white boxes plot the location of confirmed RSL (Recurring Slope Lineae) sites (McEwen et al. 2011, 2014). From Mitchell and Christensen (2016).

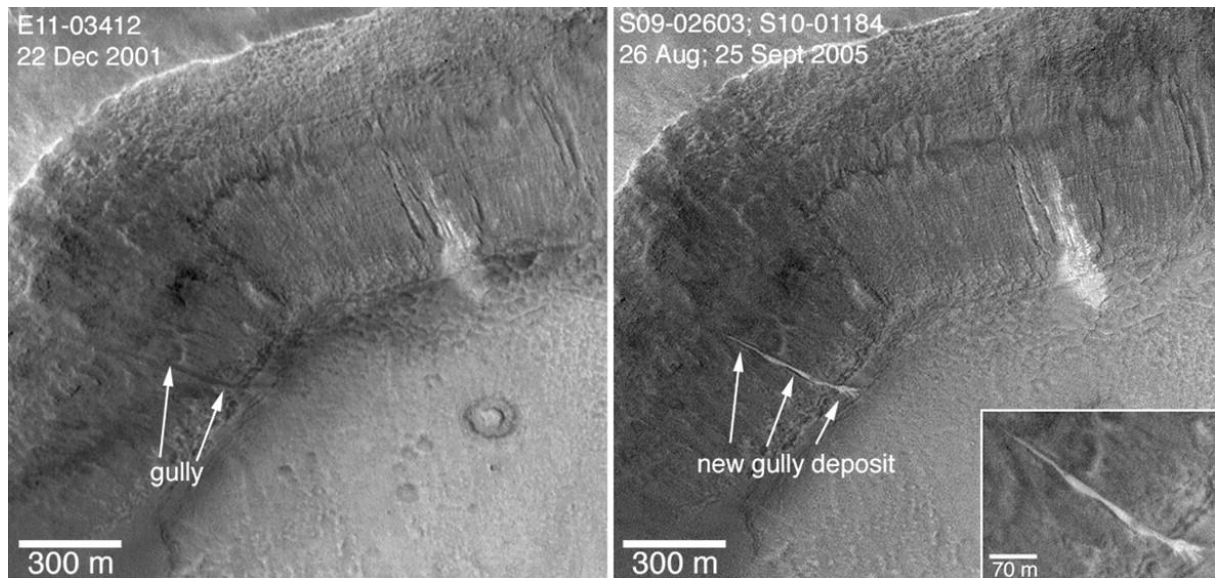


**Figure 2.3.** Detailed view of a chloride salt deposit on Mars investigated by Hynek et al. (2015). The chloride minerals are highlighted in green. Nontronite was detected in regions away from the centre, thought to be formed by aqueous alteration of the surrounding rock. The chlorides then resulted from the evaporation of this lake. Crater counting puts the age of these deposits at  $3.60 \pm 0.10$  Ga.

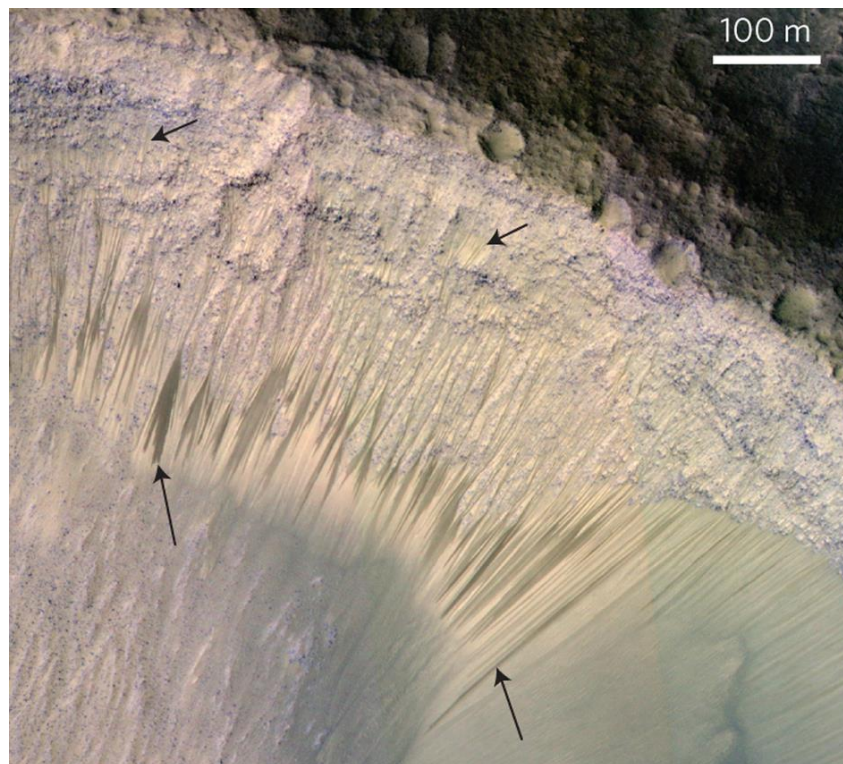
Whilst these deposits record the presence of brines in Mars' ancient history, contemporary brines may still exist on the planet. Recurring Slope Lineae (RSL) were first identified in Mars Global Surveyor Imagery Mars Orbiter Camera imagery (Malin and Edgett 2000). These features consisted of localised incised channel networks that formed on steep slopes (see Figure 2.4 and 2.5), particularly in the southern hemisphere of Mars. These features do not appear to depend on temperature (McEwen et al. 2014). A host of different hypotheses were presented to explain the RSL features, including brines (McEwen et al. 2014), dry debris flows (Pelletier et al. 2008), ground water seepage or run-off (Malin and Edgett 2000) and CO<sub>2</sub> ice freeze thawing (Núñez et al. 2016).

Recent work detected the presence of hydrated salts, most likely to be magnesium and sodium perchlorate and magnesium chlorate, in four separate locations when RSL features were most extensive. Both perchlorate and chlorate are very hygroscopic salts, meaning these streaks could be related to atmospheric moisture being absorbed by the perchlorate salts (deliquesce) until a slurry is formed which eventually becomes unstable and flows downslope. However, local aquifer discharge or the melting of subsurface ice are not ruled out as possible causes (Ojha et al. 2015). Other work found limited evidence of chloride deposits present in a small number of RSL features. The authors suggest these deposits could be explained by chloride brines being wicked to the surface and forming RSL features (Mitchell and Christensen 2016). As with the older chloride deposits, it is possible that multiple mechanisms contribute to the presence of RSL features on different parts of Mars (Ojha et al. 2015).





**Figure 2.4.** Two images taken between December 2001 and April 2005 showing evidence for active RSL features. The lighter toned deposit is  $\leq 20\%$  brighter than the surrounding crater wall and floor (Malin et al. 2006).



**Figure 2.5.** Minimum-maximum stretch infrared-red-blue (IRB) image of the south facing side of a crater found in the floor of Melas Chasma. Black arrows highlight a few of the numerous RLS features. These appear to begin in narrow channels and then splay out and finger close to their bases. From McEwen et al. (2014).

As is clear from the discussion above, evaporites and brines make up an important part of both the ancient and modern Martian environment. With the planet's now inhospitable surface conditions, the subsurface of Mars is believed to be the best place to search for life on the red planet.

The evaporite deposits observed on the surface of the planet will extend into the deep subsurface. Some RSL features may also be sourced from subsurface brine pockets. This makes it is highly likely that significant quantities of subsurface fluids on Mars would come in contact with evaporite deposits and therefore contain high levels of dissolved salts. Many of these evaporite deposits also date from periods when the planet's surface is believed to have been habitable, meaning they could act as subsurface refuges for remnant populations of microorganisms. It is clear overall that deep subsurface saline environments are highly relevant to assessing the habitability of the Martian deep subsurface and identifying the most promising regions to explore. This makes terrestrial deep subsurface evaporites promising Martian analogues.

## 2.4 Knowledge gap

Through this literature review, a range of key knowledge gaps were identified that if filled, would help lead to a significantly improved understanding of how salt shapes the deep biosphere. This thesis aims to address a number of these.

- The microbial communities examined have been predominately related to NaCl rich brines or halite. Little has been done to broaden this to brines containing other ions or different salt rocks. Given the presence of a variety of salts on places like Mars and in a range of terrestrial environments, broadening this category may open new insights into the adaptations and habitability of hypersaline environments.
- Links between brine origins and microbial communities needs to be better understood. Geochemistry and microbiology work on brines in these environments has not resulted in studies able to examine links between community composition and brine origins. The passage the brine seeps take through the deep subsurface may greatly impact both the dissolved ions in these brines and the microbial communities within them.

- Next generation sequencing techniques used to examine communities in deep subsurface evaporites are lacking from the literature. These techniques have advanced our ability to examine microbial communities significantly over the last decade. Using them to sequence whole community metagenomes would enable functional pathways to be examined along with community composition, and be compared to surface environments.
- Although some work has examined potential carbon sources related to *Dunaliella* trapped within fluid inclusions, none has looked at whether the predominately heterotrophic communities present in deep subsurface evaporites can access more recalcitrant sequences from within ancient salt or other sources entering the evaporite deposit.
- Studies have not tested for Mars relevant metabolisms, like dissimilatory sulphate or iron reduction, in deep subsurface evaporites. These metabolisms could be possible in the anaerobic Martian subsurface due to the sulphate and ferric iron minerals present.
- There is a general lack of data on the communities present in deep subsurface evaporites. Most interest has come from the study of entrapped halophiles to examine antiquity. The brines entering these deposits have received less attention. Broadening the sampling of these brines would help diversify the data set to get a more representative look at the communities present and how they compare to surface environments.

## Chapter 3: The geochemical environment of Boulby Mine

In order to understand the microbial community present within any environment, it is vital to characterise its geochemical context. This is particularly important when considering brines due to the stresses imposed by high concentrations of dissolved ionic compounds. It is also useful for helping elucidate the types of energy available to the organisms in a given environment. Furthermore, brine geochemistry can help map the origins and movement of fluids in the deep subsurface, establishing a link between the Permian salt deposit and its continuing impact on deep subsurface habitability and microbial community structure.

To better understand the geochemical context of the microbial communities present in the Zechstein evaporite sequence, a number of questions need to be answered:

- i. What is the origin and evolutionary history of the brines? Understanding the brines' journey through the deep subsurface will help tie together deep subsurface fluid evolution and its link to microbiology and habitability, improving our understanding of how local and regional geological features of evaporite sequences control the organisms inhabiting them.
- ii. What energy sources are present in the brines? Determining the types of energy sources present in the brines is important for examining how the microbial communities function in the deep subsurface.
- iii. Are there any energy sources present in the salt rocks that may have interacted with the brines? This will build on the origin and evolutionary history of the brines by determining if any of the evaporite minerals the brines interacted with contain anything that could help provide energy or carbon to the microbial community.

In order to answer these questions, a diverse set of studies were carried out. Energy sources were investigated by testing for a range of different elements and chemical species known to provide energy to microbial communities. A combination of these elemental species, isotopic measurements and regional contextual information were then used to unpick the evolutionary history of the brines. Finally, to further investigate potential sources of energy and carbon in the evaporite sequence, gas analysis was carried out to see if either were trapped in the salt rocks. XRD was also used to determine the geochemical composition of the salt rocks.

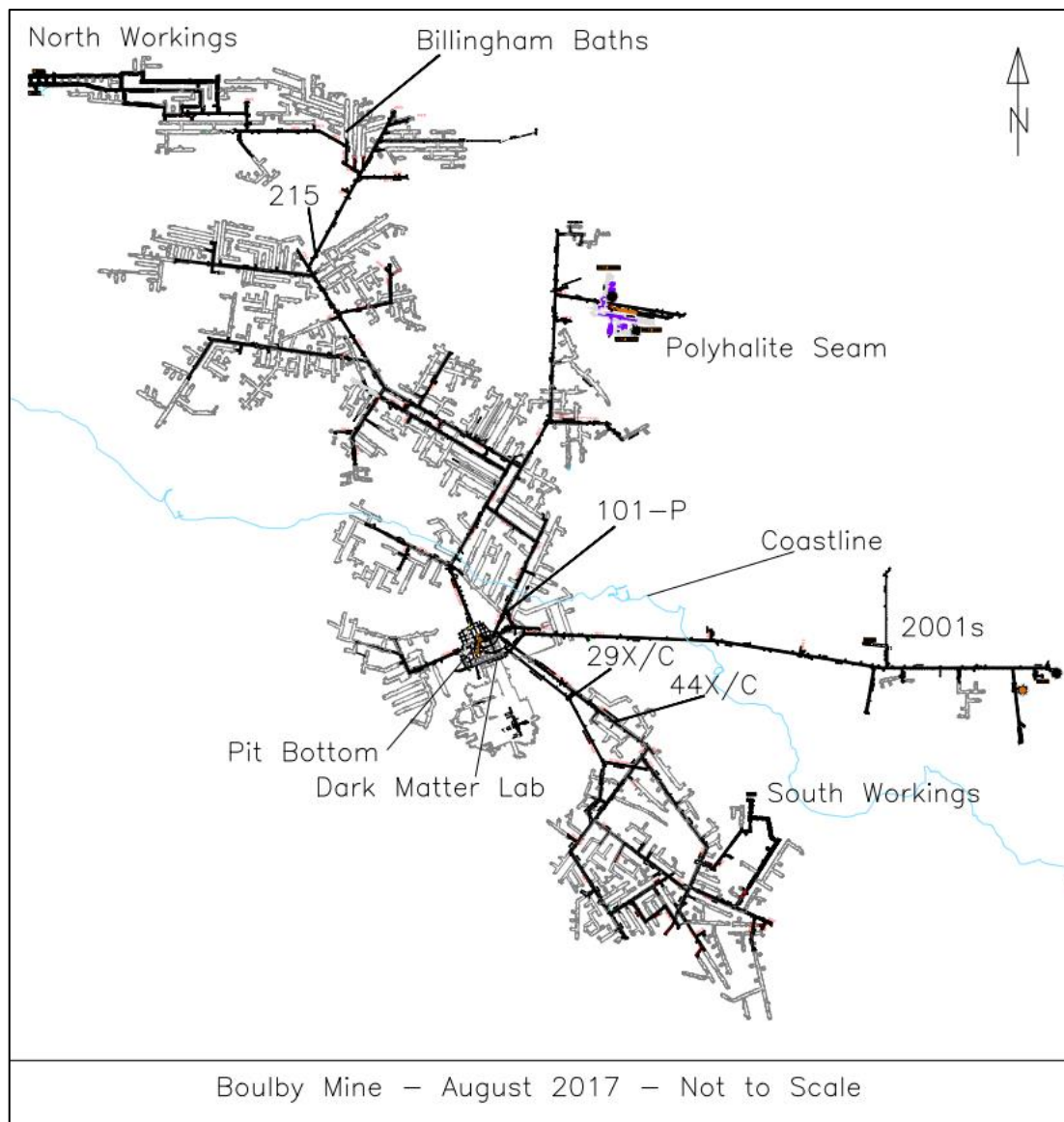
### 3.1 Field sites

As discussed in Chapter 1, Boulby mine acted as the primary field site for the work recorded in this thesis. A number of sites were sampled around the mine at different locations (described below). Initially the aim was only to examine changes in microbial communities around the mine, but this expanded into investigating brine habitability once the full geochemical composition of the brines collected was determined and the communities examined. Some of these sites are now inaccessible due to instability or changes in the mine's pumping regime.

Two main field trips were made to acquire salt rock and brine samples. The first involved sampling two brine pools/seeps (Billingham Baths and 215) collected in January 2014. The sampling strategy in this first trip was simply to acquire two brines to begin preliminary investigations into geochemistry and test DNA extraction and sequencing techniques. Therefore, the brines were chosen for their accessibility at the time of sampling and their historic longevity in the mine allowing them to be returned to should more material or additional environmental data be required. The second field trip involved sampling two brines (44 XC and 29 XC) from pump systems and another brine pool (101-P) in February 2015. The sampling strategy was more complex for this second trip. From examining a newly acquired database of brine cation composition in the mine provided by Israel Chemicals Ltd (ICL), it appeared the brines sampled during the first field trip were representative of the most common types of brine in the mine (Na/Cl brines). As the first trip had sampled brines from pools or seeps that had been exposed to the mine atmosphere for a short period of time, an additional brine with this Na/Cl composition was targeted, but one that could be sampled directly from a seep with minimal atmospheric exposure (44 XC). This would allow examination of the microbial communities before artificial oxygenation. From this database, two other brines that were atypical in composition were also selected. Such brines would have interacted with different salt minerals and may have originated from different parts of the subsurface. This may have led to them containing different microbial communities compared to the Na/Cl brines and the possibility of habitability changes if they were exceptionally high in certain dissolved ions. The locations of these brine seeps on a map of the mine is found in Figure 3.1.

Sampling the salt rocks was relatively straight forward and was carried out during both these field trips. The sampling strategy for these rocks was simply to acquire representative

samples of the major lithologies in the mine, which was achieved in consultation with the mine's geology team.



**Figure 3.1.** A schematic of Boulby mine in August 2017 with the locations of the brine seeps collected for this thesis. The coastline is outlined in light blue.

### 3.1.1 Billingham Baths brine

This is the shallowest site sampled at Boulby. It exists roughly 11 km north of the main shaft and is accessible by roadway. Since salt strata that Boulby Mine exploits dips at  $\sim 2^\circ$  to the



SE (Talbot 1982), this brine was intercepted at a shallower depth of around 700 m. As its name suggests, the area displays some of the highest fluid flows in the entire mine. The sampling site consisted of a large sump cut by the mine in order to allow the brine to collect and then pumped out to regulate water levels. Brine enters from the rear of this cavern and flows down the back walls into the main pool (see Figure 3.2). Water also pools in a ~ 3x10 m area on the right side of the wall, and flows down into the larger sump. Sampling took place as far into the brine pool as was possible to access. Given the equipment available, acquiring samples of the seep directly here was impossible due to safety issues.

### **3.1.2 Brine 215**

This exists in a small inlet on the road towards Billingham Baths at a depth of around 800 m. It consisted of a seep exiting the wall and flowing down into a sump. The brine was sampled at number of points along its flow down to the sump where it pooled in certain areas (see Figure 3.2). This region is still accessible; however, the brine has now been intercepted elsewhere in the mine meaning this sampling location is now dry.

### **3.1.3 Brine 44 XC**

Unlike the previous two brines, this brine was sampled directly from a tap that had been in place for over a year. This brine was found in the deeper, hotter southern region of the mine at a depth of around 1.3 km.

Based on the limited geochemical information available from ICL's historical records, 44 XC was selected due to its geochemical similarity to brine 215 and Billingham Baths. This would allow comparison between chemically similar brines that have pooled and become oxygenated (Billingham) or been exposed to oxygen whilst flowing downslope (215), and one sampled directly from a tap (44 XC).

### **3.1.4 Brine 29 XC**

29 XC was also sampled directly from a wall seep. The pump setup was very similar to 44 XC. This brine was much more highly pressurised than any others sampled in the mine (see Section 3.3.1.1).

29 XC was selected due its high potassium content in ICL's database, which may have meant the brine had a different formational history and harboured a distinct microbial community compared to the other brines sampled.

### **3.1.5 Brine 101-P**

101-P existed as a brine pool situated in a belt road off a main roadway in a large sump built to control water levels. Brine flowed into the pool down a ramp from the main outflow, which was inaccessible and obscured from view. The pool was approximately 5 x 10 m and over a meter deep.

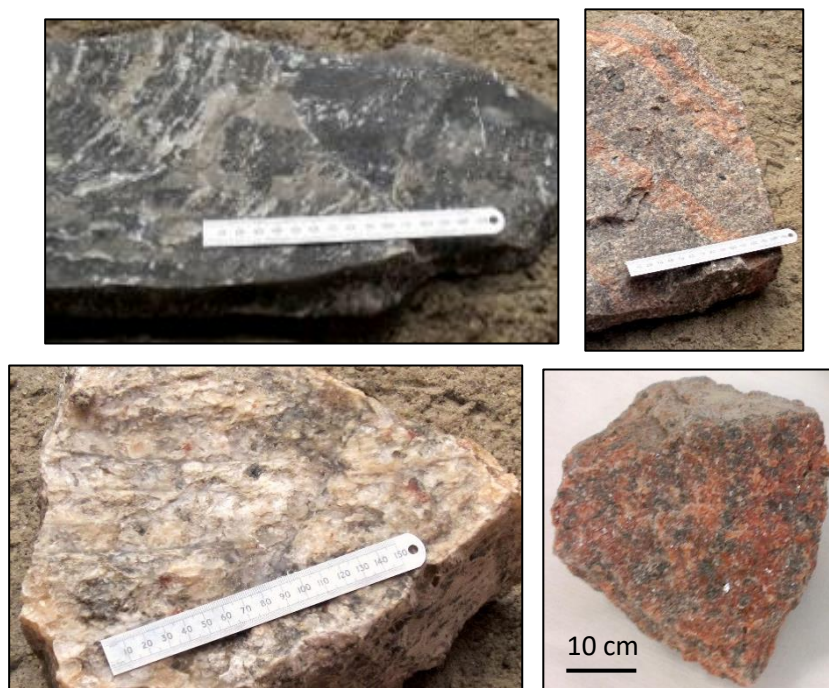


**Figure 3.2.** Contextual photographs of two brine pools sampled during this study. Top left: Main Billingham Baths brine pool in sump. Top right: Brines flowing into brine pool at Billingham Baths (person for scale). Bottom left: Close up of brine flows at 215. Bottom right: Brine flow at 215. Red colour is from the clay minerals present. 50 ml centrifuge tube (115mm in length) for scale.

### 3.1.6 Salt rocks

Salt rocks were sampled from number of different locations across the mine. The halite (NaCl) rock samples used in this study were collected from material fallen close to the Boulby Underground Facility, which is a few hundred meters from the main mine shaft. The sylvinite (a mixture of NaCl/KCl and insoluble materials) rock samples came from much deeper in the mine at the working faces. Samples were collected from blocks that had fallen off freshly mined areas. The polyhalite samples were not collected by the author, but by the geology staff at Boulby, since at the time only employees were allowed to visit that region of the mine. The polyhalite rock seam is found in the far southern reaches of the mine at depths

of close to 1,300-1,400 m, and the samples used in this study were taken from blocks extracted during the mining process.



**Figure 3.3.** Photographs of the evaporite rocks collected from the mine. Ruler in images is 15 cm. Top left: polyhalite. Top right: Banded sylvinite. Bottom left: halite. Bottom right: a different, un-banded sylvinite sample that was used for gas analysis.

## 3.2 Materials and methods

The materials and methods is split up into three main categories. The first details the techniques used to sample the brines and salt rocks from the mine. The second specifies the different analysis techniques carried out on the liquid brines and the third, the techniques used on the evaporite rocks.

### 3.2.1 Sampling techniques

The brines and salt rocks described in Section 3.1 were sampled in a number of different ways depending on the types of analysis to be carried out on them. Brines were all sampled from taps that had been in place for more than 1 year, meaning that any initial contamination had likely been diluted away.

Brines to be analysed for ionic composition were filtered through a 0.22  $\mu\text{m}$  filter into sterile polypropylene centrifuge tubes using a syringe. Those collected for cation analysis also had nitric acid added (equivalent to 1.5 ml per litre) to them to reduce the pH and prevent cations coming out of solution (Jackson 2000). They were then put into a cooler box containing ice packs and stored at 4 °C upon reaching the surface.

Samples to be analysed for TIC/TOC (Total Inorganic Carbon/Total Organic Carbon) were collected in furnace glass bottles with plastic screw cap lids. To prevent organic contamination from the lids, a barrier of furnace tin foil was placed over the top of the glass and the top screwed on over it. The glass bottles were also wrapped in tin foil to limit photochemical reactions. No headspace was included to limit mixing with atmospheric organics. These were then also placed in a cooler box immediately following sampling and stored at 4 °C once returned to the laboratory before analysis the next day.

For oxygen and hydrogen isotopes, brine samples were collected in 500 ml screw cap polypropylene bottles. These types of bottles have been shown to maintain the original  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values of water stored in them for at least 1 year (Spangenberg 2012). These were filled underwater in each brine pool or directly from a tap to the brim and screwed down, trapping as little headspace as possible to prevent isotope values equilibrating with the atmosphere. The lids were tightly sealed and then wrapped in parafilm and then tape. Samples were stored overnight in a dark cold room at 4 °C and shipped the next morning to the University of Toronto for isotope analysis.

Sylvinite, halite and polyhalite samples for XRD and gas analysis were either broken off freshly exposed faces with a geology hammer, or taken from larger pieces that had already fallen. Samples were only handled whilst wearing nitrile gloves and were transported and stored in sterile bags.

## 3.2.2 Brine characterisation

A range of data was collected to examine the geochemical composition of the brines collected, including ionic composition, temperature, ORP, pH, pressure and hydrogen and oxygen isotopic composition.

### 3.2.2.1 Brine ionic composition and TOC/TIC

The ionic composition of the brines sampled was determined using a number of techniques. Na, Mg, Ca, K and Fe were all measured using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). ICP-OES works by injecting the sample solution into a stable argon plasma contained within a magnetic field which ionises the sample. The electrons lost from the sample give off characteristic wavelengths depending on the atomic mass of element (Hou and Jones 2000). An optical spectrometer mounted is then used to record this radiation from either perpendicular (radially) or head-on (axially) to the plasma torch. This radiation is then compared to the light emitted from standards of known concentrations. Ions can be simultaneously measured over large concentration ranges using this method.

ICP-OES analyses were carried out on a Perkin Elmer Optima 5300 DV ICP-OES instrument by the School of Chemistry at University of Edinburgh. Brines had to be diluted with 18.2 M  $\Omega$ -cm distilled water from a Thermo Scientific™ Barnstead™ NanoPure™ system by factors of  $10^2$  to  $10^3$  to match the emission intensity of the standards. After dilution, Fe was the only element measured in the brines to fall below detection limits (dilution changed the Fe detection limit from approximately 0.005 mg/l to 5 mg/l). The wavelengths used to interrogate specific ions were: Na 330.237 nm, 588.995 nm, 589.592 nm; K, 766.490 nm; Mg, 279.077 nm, 280.271 nm, 285.213 nm; Ca, 315.887 nm, 317.933, 393.366 nm, 396.847 nm; Fe 239.562 nm. When using more than one wavelength per element, the wavelength with the highest  $r_2$  value was chosen to calculate the concentration of that ion in the sample.

$\text{NO}_3$ ,  $\text{SO}_4$ , Cl and Br were measured using Ion Exchange Chromatography (IEC). IEC works by utilising the natural affinity of ions to bond with an oppositely charged matrix. The strength of this affinity differs depending on the ion meaning that the rates in which they are released from the matrix differs. A positively charged matrix is used for measuring anions.

The process works by firstly loading a column containing the appropriate matrix with an anion exchange buffer containing anions not of interest in the sample (here OH<sup>-</sup> from the KOH). This saturates the active sites in the matrix. The sample to be interrogated is then loaded into the column and drawn through, with anions in the sample replacing those attached to the matrix from the buffer. At the same time, an eluent is continually drawn through the column which releases the anions in the column at differing rates depending on the strength of the affinity of the anion for the matrix. These retention times are well understood, so monitoring the conductivity of the eluent as it exists the column over time gives the abundance of specific ions. These results are then compared to known standards.

IEC analysis was carried out by the University of Sheffield by the Groundwater Protection and Restoration Group with a Dionex (now ThermoFisher) ICS-3000. Samples were filtered through a 0.45 µm pore diameter membrane and diluted 10<sup>3</sup> times with UHQ water before analysis. This brought them below the detection limits for NO<sub>3</sub> for all the brines except 44 XC. The column used was an AS18 2 x 250 mm and 34 mM KOH at 0.25 ml/min was used as the eluent. A guard column using identical packing as the analytical column was filled to prevent poisoning organic contaminants and removing particulates. A micromembrane suppresser between the analytical column and the conductivity detector is used to suppress the conductivity signal from ions in the eluent.

TOC/TIC was also carried out at the University of Sheffield by the Groundwater Protection and Restoration Group, using a Shimadzu TOC-V Series Total Organic Carbon Analyser. TOC was determined by standard procedures on the machine. This involved determining Total Carbon (TC) by burning the sample in a combustion tube to convert the carbon content to CO<sub>2</sub>, which is then examined using a non-dispersive infrared gas analyser. Inorganic Carbon (IC) is then measured by acidifying the sample with HCl down to a pH of < 3. This converts all the carbonates present into CO<sub>2</sub> which is then volatilised by purging and measured on the gas analyser. The difference between the TC and IC values is the TOC value. If some of the organic carbon is determined to be Purgeable Organic Carbon (POC), additional Non-Purgeable Organic Carbon (NPOC) and NPOC + POC methods are used to correct for this.

### **3.2.2.2 Temperature, ORP, pH and pressure**

ORP, pH and temperature were all recorded in situ using a Myron Ultrameter II™ 6Psi. The sensors were firstly cleaned with distilled water and dried at the surface. pH calibration was

done in the mine at the start of each trip using the standard pH buffered solutions provided with the instrument. All pH measurements were corrected for temperature and salinity, to keep them consistent. The Ultrameter II has an inbuilt electronic ORP calibration. Similarly, temperature calibration is not required with the Ultrameter II, although this was checked in the laboratory against a standard red alcohol lab thermometer.

To measure the pH, ORP and temperature of the brines, the sensor was firstly rinsed the brine in question three times and then refilled to dilute out any contamination. The measurements were then recorded and repeated three times, with the sensor being rinsed with distilled water and dried between samples.

Dissolved oxygen readings were taken from the brine pools using a Spectrum Dissolved Oxygen Meter (407510A). As with pH, values were corrected for salinity and temperature on the instrument. Calibration was also checked before at the surface by testing the concentration of oxygen in the air as described in the instrument manual. Due to the set-up of the taps in the mine, the brine had to be exposed to oxygen in order to extract it from the face. Therefore, it was not possible to get an accurate reading of dissolved oxygen content in the brines sampled from taps (29 XC and 44 XC). A major redesign of the pumping system at several locations would have been required to achieve this, which was not possible in this context. ORP measurements were also limited by this problem, but since they are not exclusively controlled by the presence of oxygen and tend to react more slowly to such environmental changes, they were recorded anyway with this limitation in mind.

Pressure was not measured by the author, but is reported here due to its relevance to the microbial communities discussed in later chapters. The pressure of the brines at the taps (29 XC and 44 XC) is monitored regularly by the mine for safety reasons. These values were provided by the mine from their regular monitoring two days before sampling was carried out.

### **3.2.2.3 Hydrogen and oxygen isotopes**

Meteoric waters are typically isotopically lighter than sea water with respect to oxygen and hydrogen isotopes (Craig 1961). During colder periods, such as ice ages, aquifers are recharged with waters depleted in  $^{18}\text{O}$  and  $^2\text{H}$  intruded deep into the subsurface by the overpressure generated under ice sheets (Sheppard and Langley 1984, Montañez and Poulsen



2013). This allows the evolution and origin of deep subsurface waters to be mapped through examining the ratios of  $^{18}\text{O}$  to  $^{16}\text{O}$  and  $^2\text{H}$  to  $\text{H}$ .

As discussed above, the brine was collect in Nalgene bottles and shipped to the Stable Isotope Laboratory in Earth Sciences department at the University of Toronto for analysis of these oxygen and hydrogen isotopes.  $^{18}\text{O}/^{16}\text{O}$  and  $^2\text{H}/\text{H}$  values are expressed as  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  (Equation 3.1).

**Equation 3.1.** 
$$\delta = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} * 1000 \text{ ‰}$$

$R$  = the ratio of  $^{18}\text{O}/^{16}\text{O}$  or  $^2\text{H}/\text{H}$  in the sample or standard

Samples to be analysed for  $\delta^2\text{H}$  were prepared using manganese reduction at 900 °C from a modified method by Coleman *et al.* (1982) and  $\text{CO}_2$  equilibration (see Epstein and Mayeda, 1953) used for  $\delta^{18}\text{O}$ .  $\delta^2\text{H}$  and  $\delta^{18}\text{O}$  were measured using the Stable Isotope Laboratories delta-+ XL Finnigan MAT gas source mass spectrometer and Thermo high temperature TC-EA.

### 3.2.3 Salt rock characterisation

Two techniques were used to examine the salt rocks. These were XRD to look at the mineral composition of the rocks, and trapped gas analysis to see if they contained any gases.

#### 3.2.3.1 X-Ray diffraction

X-Ray Diffraction (XRD) is widely used method of identifying the mineralogical composition of a sample. To help understand the origins of the brine geochemical compositions, it was important to determine the types of minerals present in some of the available salt rocks in the mine. XRD is also useful for linking minerology with the gas content of the salt rocks.

Samples were prepared using a number of techniques. Large lumps of rock collected from the mine were firstly crushed using a tungsten carbide mill. This was cleaned with deionised water and acetone before use. The smaller crushed lumps of rock were then added to a tungsten carbide enclosure containing tungsten carbide rings and a puck. This was clamped onto an orbital mill and ground down for 5 mins, resulting in a fine powder. The enclosure and each individual ring was cleaned separately with water and acetone between each sample.

Following this, the powder was mounted on plastic slides for the XRD. These were cleaned carefully to remove the residue of previous samples with deionised water and acetone, dried, and filled with ~1 g of salt rock powder. Careful effort was made to use as little compressional force as possible to minimise preferred mineral grain orientation. This was especially important with the salt rocks which contains anisotropic mineral grains such as clays. The samples were then placed on a conveyer and fed into a Bruker D8-Advance X-ray Diffractometer at the School of GeoSciences, University of Edinburgh. This uses a 2-theta configuration in which the X-rays are generated by a Cu-anode x-ray tube operating at 40KV and a tube current of 40mA. Diffracted x-rays were detected using a sodium iodide scintillation detector. The samples were scanned from 2 to 60 degrees two theta with a scan rate of 0.02°/second. Resultant diffractograms were compared to the International Centre for Diffraction Data (ICDD) diffractogram database library (2012 issue) using the EVA analysis package. Typically, this procedure gives a detection limit for crystalline phases of approximately 1 wt.%, although it is worth noting that XRD will only detect the mineral components present in a sample since amorphous materials do not produce diffraction patterns.

In order to quantify the amounts of the minerals present in the samples, the diffractograms were subject to Rietveld analysis using the TOPAS software package. This involved identifying the mineral assemblage present through comparing peak positions and heights with those in the powder diffraction database (as discussed above). The TOPAS program then generates a 'model' diffraction pattern, calculated from an initial estimated mineral assemblage. It then attempts to reduce the differences between the two iteratively, which typically takes around 100 iterations, until the model and observed patterns converge, revealing the amounts of the minerals in wt.%. Detection limits are around 1 wt.%.

### 3.2.3.2 Trapped gas analysis in salt rocks

The purpose of this experiment was to better understand the available energy/carbon sources available to organisms in the mine. One potential source was gases trapped in the salt crystals, which could be liberated through dissolution. Samples were collected in sterile bags from several locations around the mine (for details on sampling techniques, see Section 3.2.1). For this study, three types of salt mineral were analysed, sylvinites, halite and polyhalite.

Two techniques were attempted to liberate gases from the salt minerals, the second of which proved successful. Both began with breaking salt samples inside a sterile sampling bag into pieces <1 cm with a hammer. Briefly, the first involved placing these smaller pieces of salt into a zirconium oxide ball mill with 2 mm diameter zirconium grinding balls, fitting a gas line to one of the two valves embedded into the lid of the enclosure and vacuuming the air out and refilling it with argon gas 6 times. The ball mill was then left under vacuum for 2 hours to attempt to drive off any remaining air, and vacuum/argon flushed a further six times. It was then placed in a Fritsch P6 Planetary Ball Mill and the salt inside ground down into a fine powder at 400 rpm. Gas samples of the headspace were then taken and injected into a Gas Chromatograph (GC), as described below. This technique produced traces of many gases, but it proved difficult to break down the salt consistently and therefore accurately determining their gas contents was problematic. For this reason, a second technique was attempted.

Due to the salt being soluble in water, it was assumed that any gases present might be more easily liberated if the salts were allowed to dissolve in water and the gases left to accumulate in the headspace. This began with glass serum bottles being washed with Milli-Q<sup>®</sup> water six times and then baked at 70 °C for 20 minutes to drive off any excess H<sub>2</sub>O. Following this they were covered with aluminium foil and furnaceed at to 450 °C for 4 hours to remove any residual organics which may react and produce gas. Once the serum bottles were cooled, they were filled with the various salts (see Table 3.1).

Sample	Weight (g)	Density (g/cm <sup>3</sup> )
Halite	10	2.16
Sylvinite	10	1.2
Polyhalite	5	2.78

**Table 3.1.** Types of salt by weight investigated for trapped gases. Five grams of polyhalite was used due to its lower solubility.

Rubber bungs to seal the serum bottles were then boiled in sodium hydroxide in a fume hood for 1 hour to remove organics since they could not be furnaced. After this they were rinsed 6 times with Milli-Q<sup>®</sup> water, dried and inserted into the serum bottles and crimp sealed with an aluminium top.

The sealed serum bottles were then flushed with argon gas. Two needles were inserted through the rubber stopper, one connected to a vacuum pump and one to an argon gas canister, with a manual valve on each. The valve was then shut on the argon line and the vacuum activated. After 1 minute, the line was closed and the bottle repressurised with argon. This vacuum/argon flush cycle was repeated 6 times. Following this, each serum bottle was left under vacuum for 2 hours to attempt to drive off any remaining gases. After 2 hours of vacuuming, the bottles were again flushed with argon and then vacuumed for 1 minute 6 times, leaving each under vacuum at the end. The samples were now prepared for the introduction of the water.

To lower the risk of gas adsorption, glass syringes were used to inject the water. These syringes were firstly flushed 3 times with argon flushed Milli-Q<sup>TM</sup> water, following which 80 ml was then injected into each serum bottle which had a measured volume of 118.5 ml. To equilibrate the pressure, 40 ml of argon gas was injected to fill the remaining headspace. Serum bottles were then put in a water bath at 19 °C to keep the temperature constant whilst the salts dissolved. A blank was also included which consisted of an empty serum bottle, argon flushed water and no salt.

Dissolution was simple for the halite and sylvinite, with both easily dissolving after an hour. However, the polyhalite proved to be very difficult to dissolve. To try and encourage dissolution, three extra serum bottles containing polyhalite were left in a 20 °C ultrasonic

bath. A blank was also added due to the potential generation of hydrogen by sonolysis during this process (Ambedkar 2012). However, after a number of hours, it was clear that the polyhalite was too difficult to dissolve. Consequently, it was not used for further analysis.

Once the halite and sylvinite had dissolved, the headspace of each serum bottle was sampled. To do this, a 50 ml glass syringe was flushed with argon 3 times, then filled with 35 ml of argon. This was then injected into each serum bottle over pressurising it. The syringe was then allowed to refill itself due to the pressure difference, following which the gas was reinjected into the serum bottle 3 times to mix the headspace. Finally, 35 ml of gas was drawn out of the bottle and the syringe linked up the GC injection port. 5 ml of the gas was expelled prior to opening the port valve to remove any gas that may have mixed with the air at the end of the syringe. The rest of the gas was then injected directly into the GC.

The GC used for this analysis was an Agilent Technologies Model 7890A. Whilst the serum bottles had been under vacuum, a gas standard was injected into the GC (see Table 3.2). All samples and standard were analysed the same way, using the flame ionisation detector (FID) was used to measure CH<sub>4</sub>, CO<sub>2</sub>, ethane, propane, i-butane and n-butane and the H<sub>2</sub>, He, O<sub>2</sub> and N<sub>2</sub> were analysed on the thermal conductivity detector (TCD). The volume used for the FID was 0.5 ml and 1 ml for the TCD. Only 5 ml of gas was required to flush the gas loops in the GC, so the 30 ml of gas injected was more than sufficient to purge the system.

Gas	Concentration (ppm)
Helium	102.3
Isobutane	5.13
N-Butane	5.15
Propane	20.6
Ethane	18.87
CO <sub>2</sub>	405.6
Argon	8900
Methane	194.7
Hydrogen	493
Oxygen	20.4 %
Nitrogen	Remainder

**Table 3.2.** Gas standards used as a baseline to calculate the values of observed gases in the salt. This standard was run daily to ensure consistency. Gases not present in this standard were not examined in the samples.

Sample peak areas ( $\mu\text{volts} \times \text{s}$ ) were converted to gas concentrations (ppm) using a linear calibration equation for each gas obtained by diluting BOC 5.0 grade certified ( $\pm 5\%$ ) gas standard. These ppm values were then converted to nmol/g. The precision of the GC was measured (coefficient of variance) at 0.72 %, 1 %, 1.15 %, 7.95 %, 5.67 %, 5.52 %, 4.29 %, 2.56 %, 1.17 %, 1.25 % for CH<sub>4</sub>, CO<sub>2</sub>, ethane, propane, i-butane, n-butane He, H<sub>2</sub>, O<sub>2</sub> and N<sub>2</sub> respectively. As mentioned above, three blanks were included in the analysis. The average gas concentrations detected in blanks were then subtracted from those found in the salts.

Due to the design of the experiment, a number of corrections to the output of the GC had to be made to account for the solubility of gases in saline waters. Since the dissolution of the salt will have altered the liquid/air ratio, thereby changing the headspace volume present, it was necessary to estimate this change in headspace to understand the concentration of the

gases in the headspace when sampled. To calculate this volume ( $V$ ) shift, the equation below was used:

**Equation 3.2.** 
$$V = m/\rho$$

$m$  = Mass of the water and salt combined.

$\rho$  = Density of water containing a known concentration of a particular salt.

The density of the water with the dissolved salts was determined using data from the Mettler Toledo density concentration tables (see <http://uk.mt.com>). The halite is almost entirely NaCl, so the density for a solution of 12.5 % NaCl at 20 °C would have been used (10 g of halite into 80 ml of H<sub>2</sub>O), a value of 1.0894 g/cm<sup>3</sup>. However, a portion of this was illite (1.7 % according to the XRD data, see Section 3.3.6), which is insoluble, meaning approximately 98.3 % of the salt actually dissolved, making a salt solution of 12.29 %, giving a new liquid density value of 1.0878 g/cm<sup>3</sup>. In the sylvinite, the high concentration of insoluble illite in the sylvinite meant that only approximately 86 % of the rock dissolved, creating a 10.75 % salt solution. Additionally, this salt was a blend of NaCl and KCl due the sylvinite being a mechanical mixture of halite and sylvite (KCl). Since density tables for mixtures of salts are not available, the density value used was estimated from the combined additional densities of NaCl and KCl at 6.1 % and 4.5 % respectively, over water at 20 °C (assuming mole ratios of NaCl/KCl of 0.57/0.42 based on the XRD data for sylvinite present in Section 3.3.6). This yielded an estimated density of 1.07087 g/cm<sup>3</sup>.

It was also important to factor in the significant volume of the insoluble illite within the sylvinite and halite. XRD estimates show that illite made up around 14 % of the sylvinite rock, i.e. 1.4 g of illite at a density of 2.8 g/cm<sup>3</sup> is present per 10 g of sylvinite and 1.7 % in the halite. Since this illite is insoluble, its volumetric addition to the 80 ml of water was calculated from its weight and density using Equation 3.3. The combined volumes of this illite, the dissolved salts and the 80 ml of argon flushed water then added together and subtracted from the 118.5 ml volume of the bottle, giving the new headspace volume. Equivalent calculations were carried out on the halite to account for the 0.17 g of illite present per 10 g of halite.

Before the data could be plotted, it is also necessary to correct for the dissolution of gases released from the salt into the saline liquid. Without these corrections, the gas concentrations in the headspace would have been underestimated. To make this correction, the Bunsen

Coefficient ( $\beta$ ) was used, which is defined as the volume of a gas at standard pressure that dissolves in a solvent, when the partial pressure of the gas is equal to standard pressure (Equation 3.3) (Breitbarth et al. 2004).

**Equation 3.3.** 
$$\beta = \frac{x_i * R * T}{P_o} * \frac{D}{M}$$

$x_i$  = Gas molar fraction in the liquid phase

$R$  = gas constant

$T$  = temperature

$P_o$  = standard atmosphere

$D$  = density of water at temperature of measurement

$M$  = molecular weight of water

$x_i$  was calculated using the equation below:

**Equation 3.4.** 
$$x_i = P_i / K_s$$

$K_s$  = Henry's constant for a saline solution

$P_i$  = Partial pressure of the  $n^{\text{th}}$  gas.

To determine  $K_s$ :



**Equation 3.5.**  $\ln(K_S/K_H) = K_m * m$

To solve for  $K_S$ , this is then rearranged to:

$$K_S = \text{EXP}(K_m * m) * K_H$$

$K_H$  = Henry's constant.

$K_m$  = Salting out (Setchenow's) coefficient of a particular gas.

$m$  = Molarity of solution.

The  $K_H$  data as a function of temperature was taken from Capasso and Inguaggiato (1998) and Mohebbi et al. (2012). Setchenow's coefficient values used were taken from Morrison and Billett (1952) and Whitfield (1978). It is worth noting that the sylvinite is a mixture of NaCl and KCl and data for Setchenow's coefficient does not exist for these exact mixtures of salts. Therefore, because NaCl is dominant mineral in sylvinite (see Section 3.3.6), the values for this mineral were used.

## 3.3 Results

### 3.3.1 Geochemical composition of the brines

#### 3.3.1.1 Brine elemental composition and basic geochemical characteristics

All of the brines collected display high levels of dissolved ions (full data set is collated in Table 3.3). All are very rich in chloride ions, with the highest concentration being present in 29 XC at 210 g/L and the lowest concentration in Brine 101-P at 159 g/L. Chloride is consistently the most dominant anion present in all the brines sampled. Sulphate is the second most common anion in all of the brines except 29 XC, but is significantly less concentrated than chloride with 44 XC displaying the highest values at 3.8 g/L and 29 XC the lowest at 1.3 g/L. Instead of sulphate, bromine is the second most common anion in 29

XC at 2.5 g/L. Bromine is less concentrated in the other brines at around 0.5 g/L and 0.2 g/L in 44 XC and 101-P and below detection limits in Billingham and 215. Only brine 44 XC contained measurable amounts of NO<sub>3</sub>. However, only brine 44 XC contains significant quantities at 0.5 mg/l.

High levels of cations typically associated with chloride salt minerals were present in all the brines, although these cations differ significantly between some of the brines. Sodium was the most dominant cation in all of the brines, again except 29 XC. Billingham and 215 both contain the highest levels of Na at around 120 g/L, which are closely followed by 44 XC at around 84 g/L and 101-P with 62 g/L. Brine 29 XC contains by far the lowest amount of Na at just 9 g/L. Potassium content was also highly variable between the brines. Most display low concentrations with 101-P, Billingham and 215 all below 1 g/L. However, 44 XC and 29 XC display higher levels of K, with 44 XC at 4.8 g/L and 29 XC significantly higher at 67 g/L. This makes it the dominant cation in 29 XC and the only brine where this is the case. Magnesium content also varied between the brines. 101-P contained the highest levels of Mg at 35 g/L, followed by 29 XC and 44 XC, which contain 15 g/L and 12 g/L respectively. Billingham and 215 were both significantly depleted in magnesium, at ~1 g/L. All the brines only show trace levels of Fe.

Brine temperature varied from 29.5 °C in Billingham, up to 35.7 °C in 44 XC. It is worth noting that this temperature is strongly influenced by the surface air being forced down the mine shafts which cools the surrounding rock and liquids, to temperatures below the native rock temperature (~40-50 °C). This cooling effect fluctuates in different parts of the mine depending on the distance the air has travelled from the main shaft. Air temperatures in the mine vary from around 20 °C, up to around 50 °C, influencing the temperature of the brines when they were sampled. 44 XC for example, was sampled in the deepest and hottest areas of the mine visited during sampling, whereas Billingham is in a much shallower part of the mine closer to the start of the surface airflow.

Brine pH ranged from neutral to acidic. Billingham and 215 were both relatively neutral at around pH 7.29. Brines 29 XC and 44 XC were more acidic at around pH 5. 101-P was distinctly lower at pH 3.42.

As mentioned, pressure at sampling taps was recorded two days before sampling. 44 XC was measured at 58.5 psi (0.4 MPa). 29 XC was significantly higher at 375 psi (2.6 MPa), and effervesced fiercely when sampled. Dissolved oxygen in the brines varied slightly.

Billingham pool had the highest levels at 2 mg/l. Brine 215 and 101-P were lower at 1.1 mg/l and 0.8 mg/l respectively.

### **3.3.1.2 Hydrogen and oxygen isotopes**

The brines differed in their isotopic values (see Table 3.3). Billingham Baths and Brine 215 displayed very similar values -48 ‰ for  $\delta^2\text{H}$  in both brines and -7.4 ‰ and -7.5 ‰ for  $\delta^{18}\text{O}$  in Billingham and 215 respectively. Brines 29 XC and 44 XC contained slightly larger, but very similar isotopic values at -7.3 ‰ and -7.2 ‰ for  $\delta^{18}\text{O}$ , and -40.5 ‰ and -41.8 ‰ for  $\delta^2\text{H}$  respectively. Brine 101-P was the most enriched in  $^2\text{H}$  and  $^{18}\text{O}$ , with values higher than any of the other brines at -5.2 ‰ for  $\delta^{18}\text{O}$  and -37.8 ‰ for  $\delta^2\text{H}$ .

	F	Cl	NO <sub>3</sub>	SO <sub>4</sub>	Ca	K	Mg	Fe	Na	Br	pH	Temp. (°C)	$\delta^{18}\text{O}$ (‰)	$\delta^2\text{H}$ (‰)	Bottrell's groups	New groups
<b>Billingham</b>	b.d.	191250	<100	2045	121	971	128	<5	117396	<0.1	7.12	29.5	-7.5	-48	n/a	2
<b>215</b>	b.d.	197250	<100	1980	112	34	80	<5	120784	<0.1	7.25	32.4	-7.4	-48	n/a	2
<b>29 XC</b>	b.d.	209639	<100	1267	1315	66863	14877	<5	9174	2463	4.96	35.5	-7.3	-40.5	n/a	2
<b>44 XC</b>	b.d.	175002	560	3836	1650	4758	12384	<5	84357	523	5.15	35.7	-7.2	-41.8	n/a	2
<b>101-P</b>	b.d.	159347	<100	2545	1274	958	34604	<5	61659	233	3.42	33.1	-5.2	-37.8	n/a	3
<b>*B1 Transient flow</b>	b.d.	233000	b.d.	25000	2840	65200	29000	1.34	64000	1207	6.35	28.4	-4.8	-30	3	3
<b>*B2 Transient flow</b>	b.d.	233000	b.d.	2810	2270	71800	22500	0.45	72000	947	6.43	27.5	-5.1	-39	3	3
<b>*B3 Persistent flow</b>	b.d.	229000	b.d.	3020	2140	86700	1560	0.47	97000	263	7.5	31.4	-7.7	-47	2	2
<b>*B4 Persistent flow</b>	b.d.	118000	b.d.	3100	1290	624	452	1.21	79000	b.d.	6.85	29.2	-8.1	-50	1	1
<b>*B5 Persistent flow</b>	b.d.	121000	b.d.	4770	1970	420	660	0.36	80000	b.d.	6.77	29.0	-8.9	-51	1	1
<b>*B6 Persistent flow</b>	b.d.	218000	b.d.	3530	1330	64400	736	0.43	105000	294	6.56	28.2	-8.3	-55	2	2

**Table 3.3.** This table displays the geochemical data (in mg/l unless otherwise stated) retrieved from the brines. It also includes some data from Bottrell et al. (1996) collected from Boulby (marked with \*) for comparison. The table attempts to group some of the brines together into consistent groups based on their origins (detailed in Section 3.4.2).

### 3.3.2 Geochemistry of the salt rocks

#### 3.3.2.1 XRD

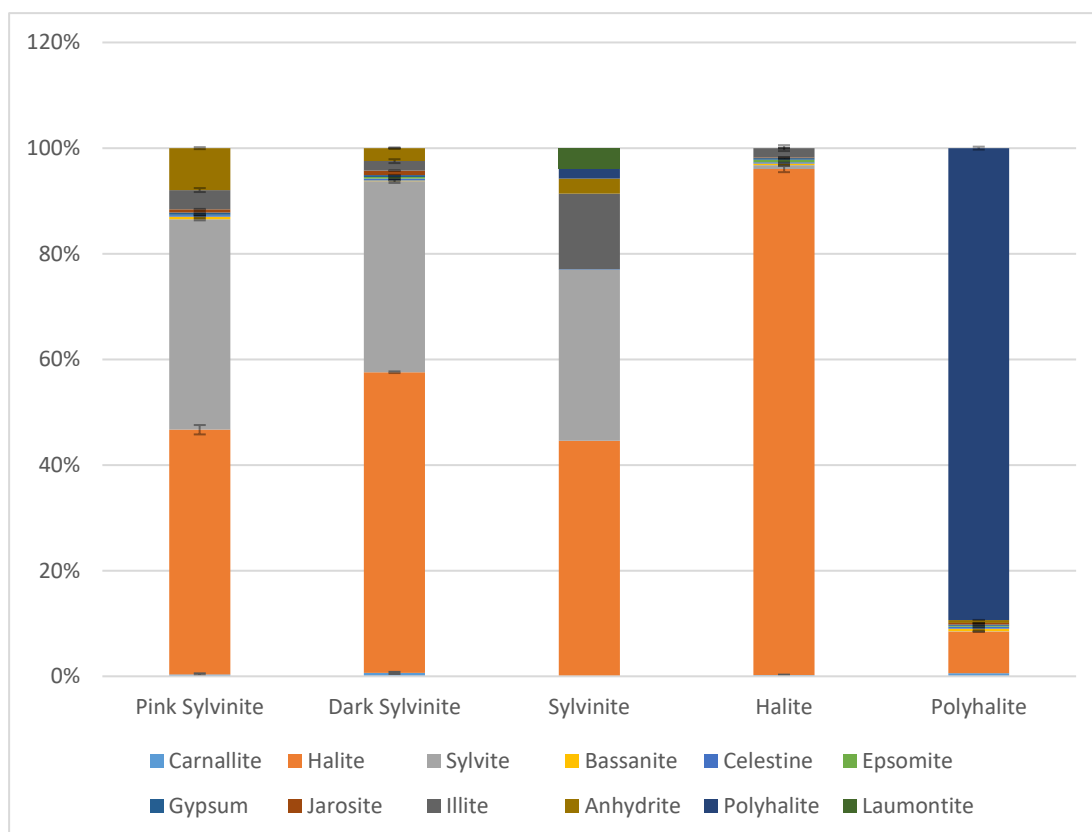
A range of minerals were identified in the salt rocks using XRD. The sylvinite rocks consistently contained the same assemblage of minerals, but the proportions of these minerals varied between different sylvinite samples. Halite was consistently the most dominant mineral, followed by sylvite (KCl). Illite

( $\text{K}_{0.6}(\text{H}_3\text{O})_{0.4}\text{Al}_{1.3}\text{Mg}_{0.3}\text{Fe}^{2+}_{0.1}\text{Si}_{3.5}\text{O}_{10}(\text{OH})_2 \cdot (\text{H}_2\text{O})$ ) makes up a significant proportion of the rest of the rock in addition to some trace mineral such as gypsum ( $\text{Ca}(\text{SO}_4) \cdot 2(\text{H}_2\text{O})$ ) and jarosite ( $\text{KFe}^{3+}_3(\text{SO}_4)_2(\text{OH})_6$ ).

The three different types of sylvinite rock (the pink layer and dark layers in the banded samples, and the un-banded sylvinite) (see Figure 3.3) appear to have some variation in the proportion of the minerals in each. The pink and dark different layers observed in the banded sylvite layers are similar in composition, with both being dominated by halite with a significant proportion of sylvite present. The main difference between the two appears to be the larger amount of anhydrite ( $\text{CaSO}_4$ ) and illite present. Both layers in the banded samples displayed lower levels of illite in comparison to the un-banded sample used for the gas analysis.

The rock described as “polyhalite” is unsurprisingly composed predominantly of the mineral polyhalite ( $\text{K}_2\text{Ca}_2\text{Mg}(\text{SO}_4)_4 \cdot 2(\text{H}_2\text{O})$ ), with a significant proportion of halite present. Similarly, the salt rock described as “halite” is also composed almost exclusively of the mineral halite, with a small amount of illite and similar trace minerals to the sylvinite.

It is worth noting that these XRD values should not be taken as exact. Although they were carried out in triplicate, positive controls (salts artificially made to a known composition) of the different salt and clay minerals were not available. This means that the precision on the measurements has been accounted for, but not the accuracy. Therefore, it is possible to compare the different amounts of minerals present in each sample within the measurement errors (i.e. x has more halite than y), but the exact proportions of salt should be considered estimates. TOPAS refinement accuracy is checked regularly using binary mixtures of minerals and results are always within +/- 10 %. Therefore, the estimates here should be within this accuracy level.



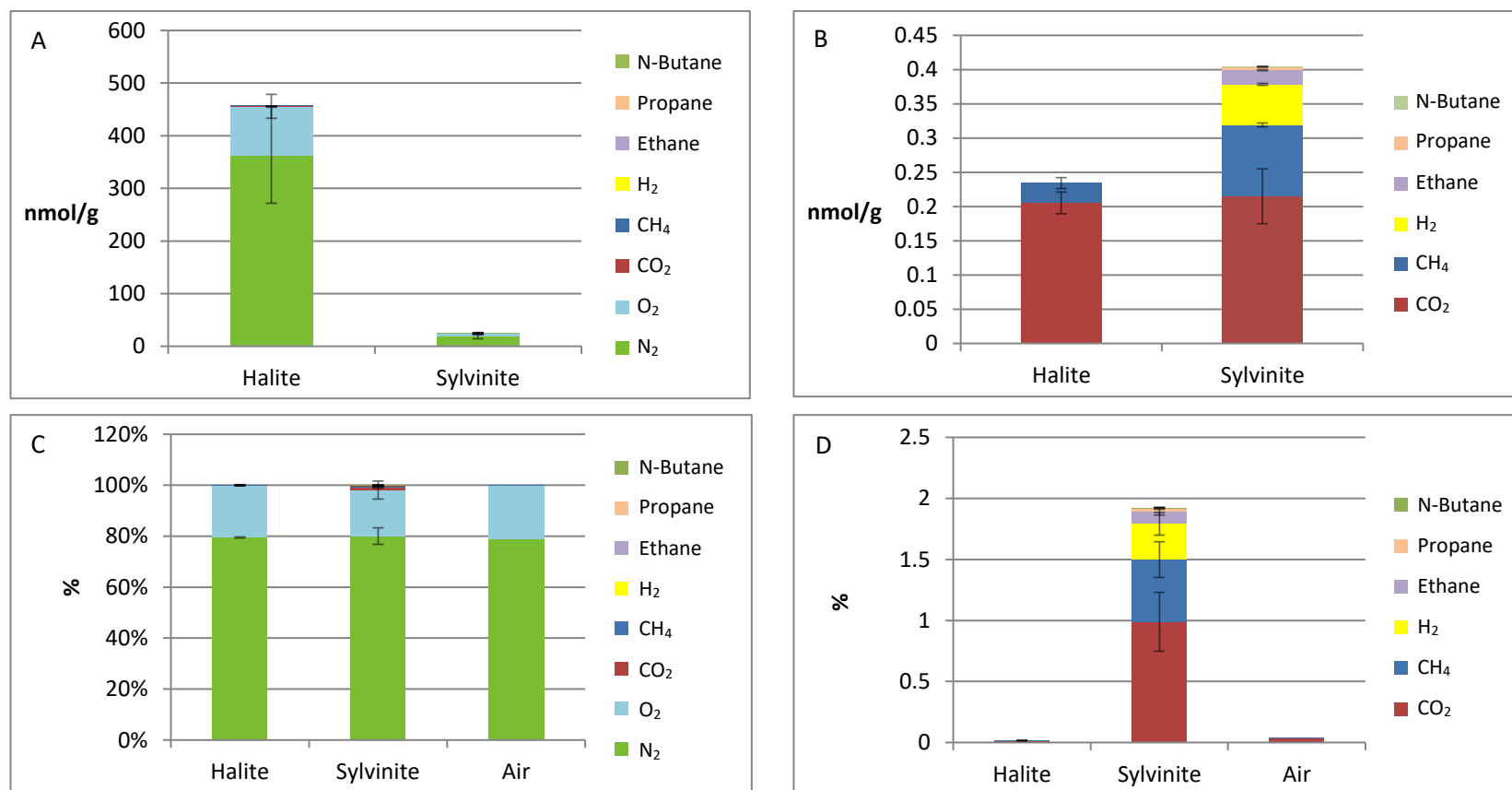
**Figure 3.4.** The proportions of different salt minerals present in pink sylvinitite, dark sylvinitite, halite and polyhalite. It is worth noting that this data was not done against XRD standards, but should be used to give a rough overall estimate of the salt minerals present in the rocks.

### 3.3.2.2 Gas analysis

Dissolving the salts in water proved to be a very successful technique in liberating gases from within the salt rock. Of the halite and sylvinitite that were tested, both appeared to contain gases. As mentioned in Section 3.2.4.2, we could not get the polyhalite to dissolve even using an ultrasonic bath and so it is not included in the results here.

The halite contained more gas than the sylvinitite in total, releasing on average of 456 nmol/g of gas, compared to 30 nmol/g in the sylvinitite. The majority of gases released from both salt rocks was  $N_2$  and  $O_2$ , much like typical atmospheric gases (see Figure 3.5). The ratio between the two gases is similar in the halite and air, but the  $O_2$  level is reduced slightly in the sylvinitite.

Although the two dominant gases are the same between the halite and sylvinite, the trace gases (here, gases that were not O<sub>2</sub> or N<sub>2</sub>) are distinct. In halite, the only other two gases present in detectable quantities were CO<sub>2</sub> (0.008 nmols/g) and CH<sub>4</sub> (0.006 nmols/g). Sylvinite also contained these two gases (CO<sub>2</sub> 0.89 nmols/g, CH<sub>4</sub> 0.45 nmols/g), with CH<sub>4</sub> making up a more significant proportion of the total trace gases. Uniquely in the sylvinite, H<sub>2</sub> at 0.26 nmols/g, ethane (0.08 nmols/g), propane (0.02 nmols/g) and n-butane (0.004 nmols/g) were all liberated from the rock. In total, these trace gases made up much greater a proportion of the total gases liberated in the sylvinite, accounting for 1.4 %, than in the halite, where these trace gases only composed 0.014 %.



**Figure 3.5.** Amount and proportions of gases trapped in the salt. A and B show the trapped gas as nmol/g and C and D show the gases as a percentage of gases expelled from the salt compared to typical atmospheric values at sea level (Schlatter 2009). A: the total proportion of gases in both halite and sylvinite. B: minor gases present only (so excludes O<sub>2</sub> and N<sub>2</sub>). C: percentage of different gases released from the two salt minerals after salt dissolution compared to air. D: the percentage of the minor gases released in comparison to air. All error bars are generated from standard error over triplicate experimental samples. Gas volumes detected in the blanks have been subtracted from the halite and sylvinite values.



## 3.4 Discussion

### 3.4.1 Origin and evolution of the brines

Combining the isotopic and ionic data from the brines with regional geochemical information makes it possible to infer the probable origins of the brines and their evolution through the subsurface. Billingham and 215 appear to be broadly similar to those in Bottrell's group (1) brines (Sherwood Sandstone waters). Billingham and 215 brines isotopically fall in the range of most modern British groundwaters (approximately -8 ‰ to -6 ‰  $\delta^{18}\text{O}$  and -55 ‰ to -30 ‰  $\delta^2\text{H}$  from Darling et al. 2003), with some potential mixing with Pleistocene groundwater (see Figure 3.6). These isotope values, combined with their geochemical makeup, suggest that they are from the same source as Bottrell's group (1), but have encountered more halite.

This hypothesis is consistent with the local geological context in which they were found. Regions of the mine which crosscut fault lines commonly show characteristic types of localised deformation in the mine roadways over long periods of time. This deformation means roadways have to be reinforced and periodically reshaped in certain areas to compensate for the movement and keep them open and stable. The area where Billingham and 215 were sampled displays some of the most extensive roadway deformation in the mine due to a large fault intersecting the mine (located approximately halfway between Billingham and 215 in Figure 3.1). This fault is associated with persistent influxes of brine, making the areas surround it some of the wettest and most active areas in Boulby. For this reason, it is assumed by the mine workers that the fault acts as a conduit for fluid movement down from the Sherwood Sandstone aquifer into the evaporite deposit. The results present here are consistent with this hypothesis. The similarity of brines 215 and Billingham to the Sherwood Sandstone waters collected by Bottrell et al. (1996) in terms of elemental profile and isotopic composition, suggests that they have exploited the fault plane, and on the way through encountered halite. One issue with this interpretation is that K and Mg levels in brine 215 and Billingham are lower than those in the Sherwood Sandstone waters. This may be related to them coming from a different part of the aquifer (see Figure 3.6) to those sampled by Bottrell et al. (1996). The fault where the brines were sampled lies around 14 km away from the main shaft where Bottrell et al. (1996) sampled the Sherwood Sandstone waters. Alternatively, the brines sampled by Bottrell et al. appear to have interacted with

some additional non-halite evaporite minerals due to their raised salinity, which may have included K and Mg rich regions. The fault conduit may have resulted in these layers being bypassed, meaning 215 and Billingham only encountered the halite layer.

In contrast to Billingham and 215, 29 XC and 44 XC both appear to have slightly more complex evolutionary histories. The oxygen and hydrogen isotopes values of these two brines still fall roughly in the range of most modern British groundwater, suggesting they are related to the Sherwood Sandstone aquifer like Billingham and 215. Their enrichment in elements such as Mg, K and Br, indicates that they have interacted with more layers of the evaporite deposit between the aquifer and the mine, as opposed to largely bypassing them through a fault line (see Figure 3.8). For example, with the sylvinite/sylvite bearing seam would account for the K, and interacting with the dolomitised shale of the Uppang Formation (Woods 1979), would account for the Mg enrichment.

Unusually, 29 XC appears to have come into contact with a significant amount of KCl, but has somehow avoided also taking up Na. This is unexpected, since the main source of KCl in the mine is sylvinite, a mechanical mixture of NaCl and KCl (see Section 3.3.2.1), meaning the brine should have a higher Na component. Pure sylvite does exist in thin very localised seams, but is uncommon, making it difficult to understand how the brine managed to take up so little Na. Brines with a similar composition as 29 XC are therefore probably quite rare in the mine. This is supported by a lack of any other brines in the database provided by ICL having a similar ratio of Na to K as 29 XC.

The isotopic composition of brine 101-P and Bottrell's group (3) brines are outside the range expected for the regional groundwater, suggesting it might be partially formed from another groundwater source. Bottrell et al. (1996) inferred that a similar shift in isotopic composition in their "group 3" brines could be explained by a mixing of Sherwood Sandstone groundwaters and "evaporite waters"; the latter referring to gypsum and other hydrous minerals waters of crystallisation and interstitial sea water. In hydrated minerals such as gypsum, water is incorporated into the crystal structure (referred to as "water of crystallisation"), which retains the isotopic characteristics of the parent water body (Sofer 1978) (assuming an equilibrium exists between the evaporate mineral that includes water of crystallisation, and the water from which it is crystallising). It is worth noting that the water of crystallisation isotopic value is fractionated away slightly from the surrounding water in the process of crystallisation. Gypsum will normally precipitate at approximately +3.5 ‰ compared to the crystallising solution which incorporate less  $^2\text{H}$ , at -15 ‰, assuming the temperature is between 18-57 °C (Gonfiantini & Fontes 1963). The Zechstein sea is thought

to have  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values comparable to modern ocean water (Bottrell et al. 1996). The isotopic composition of the Zechstein gypsum water of crystallisation will therefore likely lie between modern ocean water values and the water of crystallisation values of gypsum forming in evaporating modern ocean water (see Figure 3.6). The dehydration of gypsum through burial and heating releases these waters with high  $\delta^{18}\text{O}$  and  $\delta^2\text{H}$  values which then mix together with the ground waters lower values, resulting in a fluid isotope composition somewhere between the two end member compositions. This can also be complicated by other sources of older ground water in the region from the Permian, which can display much lighter isotopic signatures (Sheppard and Langley 1984). On Figure 3.6, brine 101-P and Bottrell's group (3) brines fall in the mixing field between the waters of the Sherwood Sandstone formation and those from an intercrystalline brine with a composition between mean ocean water and gypsum crystallisation water.

Despite the discussion above, it is still difficult to conclude that waters of crystallisation and interstitial sea water make up part of 101-P. The evaporite sequences experienced a maximum burial depth of >2.2km during the Jurassic/Cretaceous (Talbot et al. 1982). This makes it likely that the sequence would have been exposed to pressures and temperatures sufficient for these waters to have been expelled and substantially diluted. This circumstantial evidence makes it hard to isolate a source for 101-P, but possible to conclude that its source is distinct from the other brines collected.

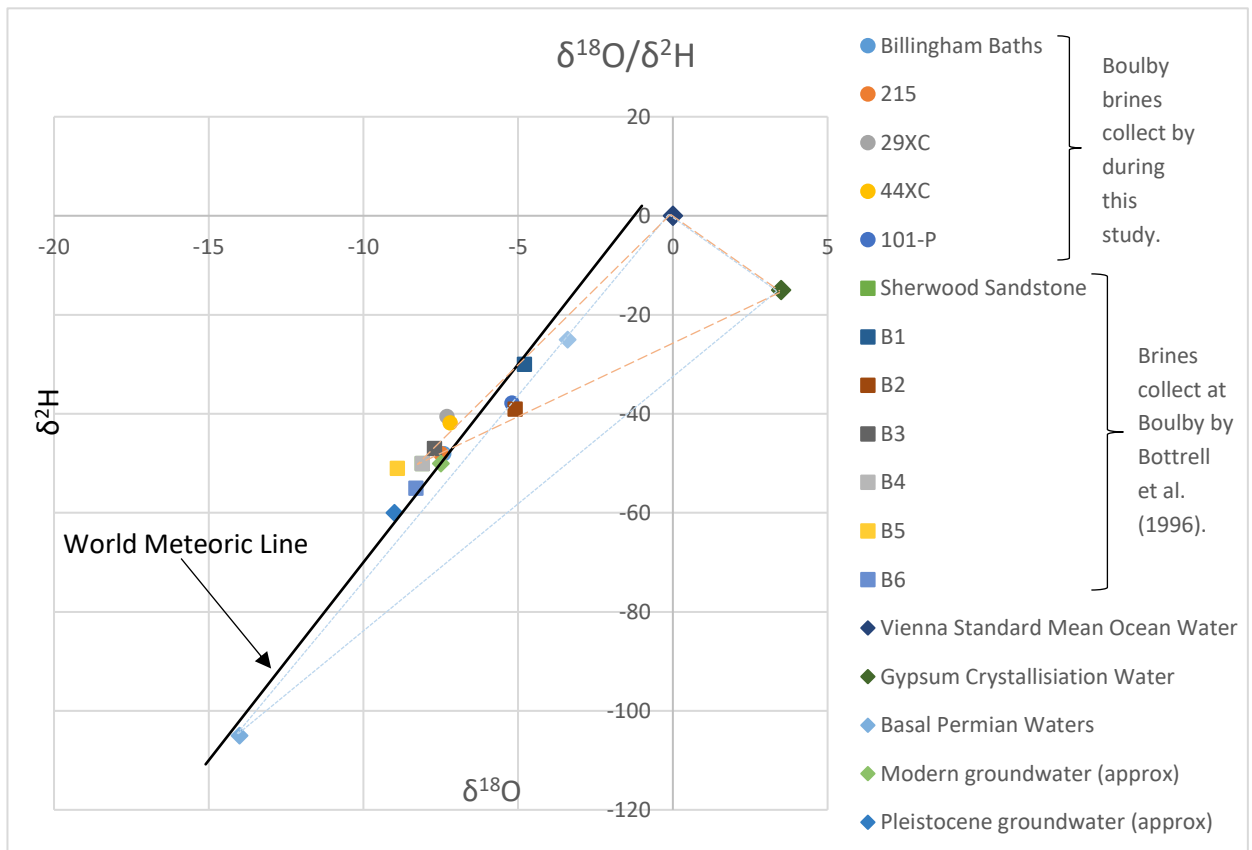
The Billingham and 215 brines clearly demonstrate that brines can travel down into the evaporite deposit from regions above the low permeability marl. However, there is evidence that fluids are also able to travel up from regions below the mine. The hydrocarbon samples discussed in Chapter 6, were collected from hydrocarbon seeps (often found with brine seeps) that periodically appear in the ceiling of the mine. These hydrocarbon seeps have most likely originated from the carboniferous coal measures below the mine, indicating upward fluid movement is also responsible for some of the brine seeps in the mine.

Overall, the brines have come from two major sources, the Sherwood Sandstone aquifer (Billingham, 215, 29 XC, 44 XC and brine groups (1) and (2) from Bottrell et al. 1996) and a distinct source (101-P and group (3) brines from Bottrell et al. 1996). Despite the common origin of the brines from the Sherwood Sandstone aquifer, their geochemical variety suggests they have had different evolutionary histories once they left the Sherwood Sandstone aquifer.

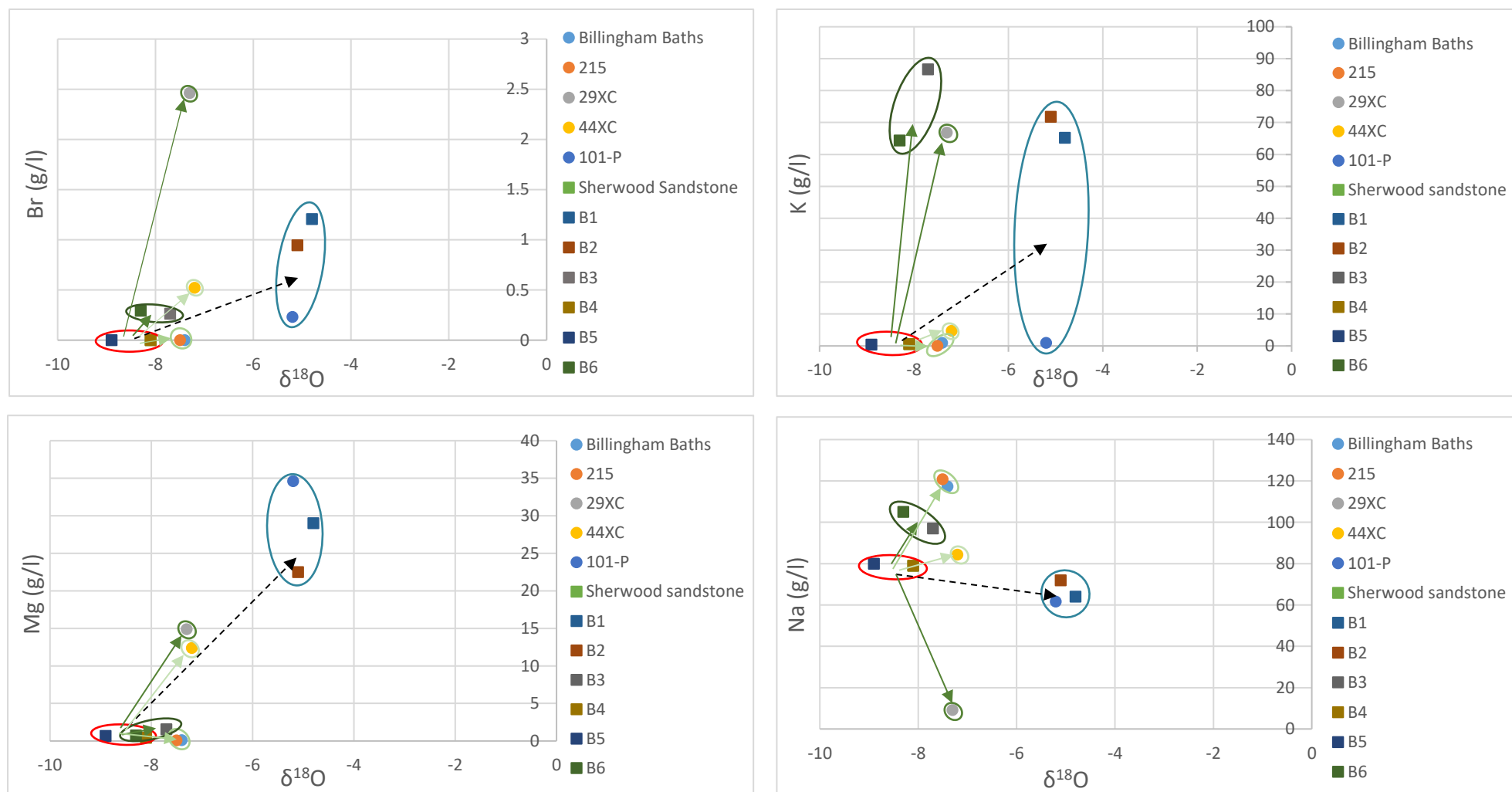
### 3.4.2 Grouping the brines

To help establish commonality between the brines to identify those with similar formational histories, it is useful to try and group them. Bottrell et al. (1996) grouped brines sampled in Boulby into three categories based on their geochemical makeup. Group (1) were sampled from the Sherwood Sandstone and were described as being dominated by Na and Cl ions, but at roughly half the concentration of the other NaCl dominated brines (~120 g/L of Cl). They also have low K in relation to Na (~0.007), a lack of detectable Br and low Mg. Group (2) brines were found as inflows in the mine and are more concentrated in Cl (220 g/L), have a higher K to Na ratio (~0.8) and are enriched in Br relative to group (1), but still have low Mg. Group (3) brines were also found as inflows, but present a distinct chemistry from group (2), with very high Mg content, further Br enrichment and a greater K/Na ratio (~1). They are also highly enriched in  $^{18}\text{O}$  and  $^2\text{H}$  compared to groups (1) and (2).

It is possible to match some of the brines collected during this study to the groups established by Bottrell et al. (1996). However, the brines collected during this study show there to be more geochemical diversity present within these groups (see Figure 3.7 and Table 3.3). The establishment of three other brine groups is justifiable based on their geochemical profile in addition to the three outlined by Bottrell et al. These being, an additional group for Billingham and 215 which are generally similar to the group (1) brines from Bottrell et al. but have higher concentration of Cl and Na and are more depleted in K. Two separate groups for 44 XC, with its enrichment of  $\text{NO}_3$  Mg and Br, and 29 XC with its exceptionally high K and low Na content. 101-P is very similar to the group (3) brines defined by Bottrell et al. so could be included in that group. However, attempting to group 44 XC, 29 XC, 215 and Billingham using geochemical profile rapidly becomes convoluted and results in nearly as many groups as brines analysed. It is much simpler therefore to group the brines strictly by their proposed origins. With this definition, 44 XC, 29 XC, 215 and Billingham could be placed in the same group as Bottrell's group (2) brines and 101-P into group (3) (assuming 101-P and group 3 share similar origins). Group 1 brines are the original Sherwood Sandstone waters. This geochemical diversity within the group (2) brines highlights the variety of brine compositions present within the Zechstein sequence, even when brines originate from the same source region.



**Figure 3.6.** Isotopic data from the brines collected during this study and by Bottrell et al. 1996, plotted with the world meteoric water line, Standard Mean Ocean Water (SMOW), gypsum crystallisation water from Standard Mean Ocean Water and other British groundwater. Two theoretical mixing fields have been added. Both draw a line between standard ocean water and gypsum crystallisation water compositions (since gypsum water of crystallisation isotopic composition will be some mixture of the two). The dashed line indicates a mixing of a gypsum crystallisation water/SMOW, with meteorically derived groundwater (Sherwood Sandstone waters). The dotted line does the same, except with the isotopically light Permian groundwater taken from Sheppard and Langley (1984). Modern and Pleistocene ground water approximate values are from Darling et al. (2003). Gypsum crystallisation water from Bottrell et al. (1996).



**Figure 3.7.** Oxygen isotopic profile plotted against four cations which help distinguish the brines. The dashed lines represent a hypothetical mixing line between the group (1) and group (3) brines. Solid lines of different shades of green join the smaller group (2) sub-groups, with the Sherwood Sandstone waters (group 1) (not fluid mixing lines), highlighting the variety of brine geochemistry in a small region of the Zechstein sequence, even amongst fluids with the same origin. Circle markers are plotted from data taken during this study and square markers are taken from Bottrell et al. (1996).

### 3.4.3 Origin of the gases and entrapment mechanism

The gas analysis carried out on the salt rocks shows that potential energy sources exist trapped with certain salt minerals in the deep subsurface. The halite for example, contained a very small portion of methane. Since the composition of the gases within the halite are similar to those of normal atmospheric air, additional work would have to be carried out to identify the source and age of these gases to determine if they are actually present in the deep subsurface without contamination from the mine air.

The air in the sylvinite is not of typical atmospheric composition. The trace gases present in the sylvinite (see Figure 3.4) do not exist in the quantities found in normal atmospheric air, suggesting the rock has been exposed to these gases some point in its geological history. The combination of molecular hydrogen, methane, ethane, propane and n-butane is typical of natural gas expelled from a hydrocarbon deposit (Selley and Sonnenberg 2014). However, to determine whether these gases were produced biologically or thermally, the ratios of particular gases need to be examined. It is worth noting that some evidence exists also to show that larger hydrocarbon molecules ( $C_2$  to  $C_4$ ) can be produced biologically (Schulz and Zabel 2006), so the presence of these larger molecules does not rule out a biological source. A key distinguishing feature between thermal or biological light hydrocarbons is the ratio of methane to ethane and propane. Typically, biology produces methane in far greater quantities than ethane or propane, with a ratio ( $C_1/(C_2+C_3)$ ) that can exceed  $10^3$  to  $10^4$ . However, in thermogenic gas, that is far lower, at less than 100 (Bernard et al. 1976). The  $C_1/(C_2+C_3)$  ratio present in the sylvinite is 4.6. This strongly suggests the gases have a thermal origin, potentially as petroleum gases from the underlying carboniferous layers.

It is worth examining why only the sylvinite contained the petroleum gases, but the halite did not. The halite layer is further down the sequences and therefore closer to the carboniferous strata making it closer to the probable source of the gases. Additionally, the sylvinite does not seem to have a higher porosity, due to the smaller total amount of gas that was released from it in comparison to the halite. This difference could be due to sylvinite existing directly below the low permeability marl layer (see Chapter 2). Gases travelling up from deeper down may accumulate in the regions below the marl and have time to enter the pore spaces in the sylvinite. However, a better explanation supported by other research is that the sylvinite is more prone to holding on to gases due to its clay content. Adsorption of  $CH_4$  has been shown to occur in clay-rich rocks (Zhang et al. 2012). As discussed, illite is present in the sylvinite rock. Although illite is one of the clays less prone to gas adsorption, it does still

exhibit the behaviour (Zhang et al. 2012). For this reason, both the halite and sylvinite may have been exposed to influxes of these hydrocarbon gases, but due to the lack of clay in the halite, the gases were not retained.

If the clays are responsible for the sylvinite retaining the light hydrocarbons, then the effect of this adsorption needs to be taken into account when discussing the ratios of the hydrocarbons present. Cheng and Huang (2004) showed that different types of hydrocarbons are preferentially absorbed by different clay minerals depending on their vapour pressure. Heavier gas molecules appear to be more readily adsorbed into the clays. This suggests that the gases trapped in the salt contain less methane and more ethane, propane and butane than the original gas. However, even in the most strongly adsorption selective clay/carbon source used in the Cheng and Huang study (active carbon), the methane/ethane ratio was only reduced by around 77% (~0.13 to ~0.03 in the initial gas mixture). If we consider this as the most extreme scenario for the selective adsorption occurring in the sylvinite (data was not available for illite), then the methane ratio would still remain comfortably in the range of thermogenic gases and therefore is unlikely to be of biogenic origin. Since it is a common gas produced in hydrocarbon deposits, the hydrogen found may have also originated from the underlying carboniferous deposit. Radiogenic hydrogen production by the decay of  $^{40}\text{K}$  in the sylvinite might be a possible source, although the decay would have to be producing more hydrogen than was diffusing out of the salt. However, due to the presence of the other hydrocarbon gases, it seems more likely that the hydrogen shares a common origin with them.

As shown in Section 3.3.2.1 (sylvinite), there is a considerable difference in the amount of illite present in the sylvinite samples. This will have affected the amount of gases trapped in the rock meaning not all of the sylvinite will display these levels of trapped gas. Further work could be carried out on these samples to determine if there is a correlation between gas content and clay content.

### **3.4.4 Implications for biology**

The geochemical composition, origin and evolution of the brines could have significant influences on any microbial communities present within them. As discussed in Chapter 2, survival in a hypersaline environment requires the expenditure of high levels of energy (Oren et al. 2011b). Only the coupling of certain oxidised or reduced chemical species can produce enough free energy to provide organisms in hypersaline environments with the energy



required to survive. Of these metabolisms, the most energy favourable is the coupling of oxygen and organic carbon. In low oxygen scenarios, a number of other less energy favourable electron acceptors are present in the brine.  $\text{NO}_3$  was only detected in 44 XC, but due to the dilution required for analysis could have been present in trace quantities in all the brines.  $\text{NO}_3$  has been shown to be used by halophiles living in chloride brines through processes such as denitrification (Oren et al. 2011b), so could provide an alternative electron acceptor in when oxygen is absent. Although it is less energetically favourable than  $\text{NO}_3$ , the  $\text{SO}_4$  present at significant levels in all the brines could also be used as an electron acceptor. A number of sulphate reducing organisms are halophilic (e.g. Ollivier et al. 1991, Jakobsen et al. 2006) and may therefore be present in the brines.

TOC/TIC measurements show that the brines from the taps contain concentrations of organic carbon slightly lower than typical rivers (TIC/TOC, 29 XC 4.06/6.8 mg/l, 44 XC 5.43/8.2 mg/l, 101-P 0.85/20.3 mg/l). These values are high for typical ground waters which are on average around 0.7 mg/l (Thurman 2012), and could be related to influxes of material from the carboniferous layers below the mine. This is particularly clear in brine 101-P, which appears to have travelled up from layers closer to the carboniferous layers (see Section 3.4.1) and may also be more effective at preserving organic matter due to its high levels of  $\text{MgCl}_2$  and chaotropic properties (Hallsworth et al. 2007, Polymenakou et al. 2007) (see Chapter 4). This organic carbon could provide a plentiful electron donor for the communities present in the brines.

Air is forced into the mine constantly to provide the workers with safe working conditions. This means that the brines which have been allowed to pool have taken up oxygen from the surrounding air, artificially providing the communities within with an oxygen supply. Dissolved oxygen readings from the pools vary, showing levels ranging from around half saturation levels (Billingham) to bordering on anoxic in 101-P. The true levels of oxygenation in pristine brines unaltered by the mine air would be useful to obtain, but it is possible to speculate that due to the depth the brines were sampled at, it is highly likely that their oxygen content is greatly reduced.

The variety of pathways travelled through the deep subsurface by the brines means they have encountered a range of different evaporite minerals. This could have a significant impact on any microbial communities present in the brines for several reasons. The ionic profile of the brines varies widely, even amongst those sourced from the Sherwood Sandstone. These differences in ionic profile may select for different organisms or even render certain brines uninhabitable (see Chapter 4). Additionally, the gas analysis showed that that certain

evaporite minerals trap gases. The hydrogen and gaseous hydrocarbons detected in the sylvinite could act as an energy/carbon source for deep subsurface life and evaporite deposits in general. A brine with a high KCl content such as 29 XC has clearly interacted more with the sylvinite layer than Billingham for example. This means it may have encountered evaporite minerals containing these gases which could have important consequences for the microbial communities present. Clay content seems vital for trapping these gases and could therefore be key to the delivery of energy and carbon to deep subsurface organisms in these types of deposits. However, data in Chapters 5 suggests the organisms in the brines might not be able to utilise these gaseous hydrocarbons.

### 3.5. Conclusion

The aim of this study was to characterise the geochemical environment in Boulby mine, determining the origin and history of brine seeps and identifying some of the energy and carbon sources present in the brine and salt rocks. This was achieved through a diverse set of techniques. The following conclusions can be drawn from this work:

- The brines have clearly been sourced from two main regions in the deep subsurface, the Sherwood Sandstone aquifer and another source distinct from the regional groundwater.
- The brines have taken a diverse set of pathways through the deep subsurface which have proven highly influential on their composition at the point of sampling. Most are dominated by Na/Cl, but brine 29 XC is dominated by K/Cl and 101-P, Na/Mg/Cl.
- A number of different energy sources are present in the brines.  $\text{NO}_3$  and  $\text{SO}_4$  are found in a number of brines which can be used as an electron acceptor in the place of oxygen by some organisms. The former is particularly relevant if trace quantities are present due to the highly exergonic nature of some dissimilatory nitrate reduction methods that have shown to be able to function at NaCl saturation. Organic carbon and molecular hydrogen in the salt rock are also present as potential electron donors.
- Salt rocks with high clay content capture natural gases migrating through the evaporite deposit. Evidence from the geochemical composition of the brines

indicates some have interacted with these clay rich sylvinite rocks, meaning during dissolution, these gases would have been released and could therefore provide energy and carbon sources to deep subsurface organisms.

Whilst part of this chapter's aim is to provide context for the chapters which focus on the microbiological content of the brines, new information about these deep subsurface environments has been gained from examining the geological environment in detail. Further work at Boulby should involve determining a way of sampling brine oxygen content accurately, whilst generally increasing the number of brines sampled in different locations around the mine. This would generate a comprehensive data set enabling statistical analysis of where the majority of brines in the mine are sourced from, and the different pathways they take through the deep subsurface. More broadly, further work in other evaporite deposits around the world should examine the gases in rock salt containing different levels of clay to see if such links between carboniferous and Permian evaporites are common, and how this might influence the communities within.

## Chapter 4: Does brine habitability vary in the Zechstein evaporite sequence?

Chapter 3 demonstrated brines in the Zechstein sequence display a range of geochemical compositions over a relatively small spatial scale. As discussed in Chapter 2, different levels of ions can result in fluids with physicochemical properties that render them uninhabitable by limiting or impairing biological functions (see Section 1.2 for habitability definition). Understanding more about the physicochemical conditions of fluids and how they influence habitability and limit the biospace will be key in examining life in extraterrestrial environments such as Mars, where highly saline fluids could be common. It will also assist in better defining the limits of habitability and its implications for terrestrial environments. A habitable environment is defined here as an environment capable of supporting the activity of one or more known organisms (Cockell et al. 2016). Using the work from the previous chapter on brine origins, this chapter aims to establish a link between the Permian salt deposit and its continuing impact on deep subsurface habitability. To achieve this, a number of questions need to be answered:

- i. Is it possible to grow organisms in the brines? This will provide a direct examination of brine habitability through enrichment techniques.
- ii. Can the brines be used to start microbial cultures? This will again examine their habitability, giving some indication of whether the brines harbour any organisms capable of growing once out of the brines.
- iii. What physicochemical properties impact the habitability of the brines?
  - o Is the water activity of the brines too extreme for life? Water activity is a key proxy for habitability and has been demonstrated to limit microbial propagation in a number of environments, including brines. It has also been suggested to limit life in Martian environments.
  - o What are the chao/kosmotropic properties of the brines? Chaotropicity is thought to limit habitability in some brine environments where other stressors are benign.
  - o What is the ionic strength of the brines? This has recently been shown to limit microbial propagation in specific Mars relevant brines (Fox-Powell et al. 2016).

- Could the temperature, pH or pressure limit microbial activity by combining with other stressors? Data related to these parameters was detailed in Chapter 3, showing all three to be individually benign. However, they could still have an impact on habitability if acting in concert with other stressors.
- iv. What are the links between brine habitability and their origins and evolution?

To answer these questions, two sets of experiments were carried out. Enrichment was attempted directly in the brines using several inoculums to see if growth would occur. Additionally, aliquots of the brines were taken and a variety of media used to try and enrich organisms. The brines were then examined using a range of different assays to measure proxies for habitability in order to determine why certain brines did or did not appear to be habitable in the enrichment experiments.

## **4.1 Materials and methods**

The habitability of the brines was examined using a range of culturing techniques. Additionally, to identify any aqueous geochemical features that might impose stress on biological systems within the brines, a range of brine physicochemical parameters were measured to understand the reasons for any changes in habitability.

### **4.1.1 Sampling techniques**

Brines for water activity and chaotropicity assays were sampled by collecting brine in sterile 50 ml centrifuge tubes and placing them in a cooler. They were then stored at 4 °C. Before any measurements were taken, the brines were checked to see if any crystals had formed at the bottom of the tubes. If crystals were present, the brine was heated to 60 °C until the crystals dissolved and then cooled back down to room temperature before any of the assays began. Soil was collected for use as an inoculant using sterile centrifuge tubes from outside the James Clark Maxwell Building, Kings Buildings Campus at the University of Edinburgh. This was collected on the same day the enrichments were set up.

Brine to be used for aerobic culturing (both as an inoculum and as a media) were collected in sterile 50 ml centrifuge tubes from their respective locations around the mine (see Chapter

3). Those to be used for anaerobic culturing were added to sealed sterile anaerobic serum bottles containing L-cysteine-HCl (a reducing agent), which once filled resulted in a final concentration of 0.8 mM. They were then immediately placed into a cooler box to reduce microbial activity and prevent growth. Once at the surface, the brines were stored at 4 °C to prevent the community changing before culturing.

## 4.1.2 Culturing

To test the habitability of the brines directly, several methods were used to grow organisms from the brines, and in them.

### 4.1.2.1 Enrichment in the brines

Brines 44 XC, 215, Billingham, 29 XC and 101-P were all used as a base media for this experiment. Culturing was attempted in both aerobic and anaerobic conditions. In both conditions, brine used as media was filtered through 0.45 µm syringe-driven polycarbonate filter units (Merck Millipore, Massachusetts, USA) to remove the clay minerals present in the brine. These heavily interfere with microscopy and spectrophotometry by binding with the nucleic acid stains and then generating large amounts of background fluorescence when examined under particular wavelengths, making it hard to visualise microbial growth.

#### 4.1.2.1.1 Initial enrichments

A set of enrichments were carried out using two carbon sources in aerobic conditions. These were designed to eliminate a number of habitable brines so that more extensive enrichment work could be carried out on any which failed to support growth.

The two carbon sources used were yeast extract and a mixture of sodium pyruvate and casamino acids. Yeast extract was chosen due to the range of amino acids, proteins, sugars and other trace elements present within it. Sodium pyruvate and casamino acids were used to broaden the range of different types of carbon sources. Pyruvate for example appears to be an important carbon source in halophilic communities (Oren 2015b) and some halophiles have very specific carbon requirements such as *Halosimplex carlsbadense*, (Vreeland et al. 2002), which was isolated from a salt mine. These two carbon sources were seen as providing an adequate examination of brine habitability and similar methods have been used

before to examine extreme brines (Fox-Powell et al. 2016). Water activity was remeasured and found to be identical after adding carbon sources (see Section 4.1.4).

The aerobic enrichments were carried out as follows: 500 ml of filtered brine was measured into Duran bottles. Two sets of carbon sources were then added to the brines as powders, yeast extract at a concentration of 4 g/l and a mixture of sodium pyruvate and casamino acids at concentrations of 1 and 2 g/l respectively. The Duran bottles were then sealed and autoclaved at 121 °C for 20 minutes.

Once cooled, a 50 ml aliquot of brine/carbon source mixture was then transferred to 200 ml glass conical flasks which were sealed with a foam bung wrapped in tin foil inside a laminar flow hood using a 25 ml serological pipette and Eppendorf Pipet Helper ®. The unsterilised brines were then removed from the fridge and mixed by inversion. At the time of culturing the brines had been stored in the fridge at 4 °C for < 2 weeks. Five hundred microliter aliquots were removed from each unsterilised brine using a pipette gun with a sterile tip and inoculated into the sterilised brine/carbon mixture (of the same brine) in the conical flasks. Each enrichment condition was carried out in triplicate to maximise the chances of a positive enrichment. These flasks were then placed in a shaker incubator at 37 °C (typical optimum temperature for cultivating Halobacteriacea, e.g. Rodriguez-Valera 1995), at 80 rpm and growth examined over a 30-day period. This temperature combined with the abundant carbon sources aimed to increase the energetic favourability of cellular respiration and maximise their chances for dealing with the stress imposed by the brines (Oren et al. 2011).

#### *4.1.2.1.2 Examining growth*

Microbial proliferation was tested by optical density. If this did not result in visible growth, brines were additionally examined with microscopy to see if low levels of growth could be identified in comparison to the starting concentration and the blank. Blanks consisting of just the carbon source and sterilised brine were also set up identically and incubated to ensure clouding was not related to geochemical processes.

Microscopy utilised florescent nucleic acid stains. 200 µl samples of brine cultures were mixed with 1x SYBR Gold acid stain (Life Technologies, Carlsbad, California, USA) and placed in a dark box for 15 minutes. They were then filtered through a 25 mm black polycarbonate 0.22 µm filter (Merck Millipore, Massachusetts, USA) and mounted on a glass slide. A wavelength of 450-490 nm was used to excite the dye and the results examined through a LP515 ( $\geq 515$  nm) emission filter using a Leica DM4000B digital microscope at

1000x magnification. Any images recorded were taken using a Leica DFC 450 C camera mounted on the microscope.

#### *4.1.2.1.3 Expanding enrichment conditions*

The results of the initial experiments above (see Table 4.3), revealed a number of brines to be habitable. This allowed those which showed no growth to be down selected and subject to a wider range of enrichment cultures to increase the chances of a positive enrichment. This involved inoculating the brines with the community from other brines (e.g. inoculating sterile 101-P with a raw environmental sample of 215). A 500 µl (or 1 g of soil) inoculation into the sterile brines containing the carbon sources was used for this. The rationale behind using the communities from the other brines as inoculum covered the possibility that certain brines may be habitable, but did not contain any viable organisms. In addition to this, aliquots of enrichment cultures (see Section 4.2.1) using the same carbon sources were added into the brines to see if growth would occur. This was carried out in case being stored in the fridge compromised their ability to colonise the brines.

Anaerobic enrichments of the brines which failed to produce growth were also set up using these carbon sources. Triplicate 50 ml samples of each brine/carbon source mixture were added to a 200 ml serum bottle and crimp sealed with a rubber butyl stopper and aluminium top. These were then sparged with nitrogen gas, filtered through a 0.2 µm syringe filter using a sterile needle and syringe attached to a gas station. The tops of the stoppers were sterilised using ethanol and flamed, and the remaining manipulations carried out under a Bunsen flame. After sparging, the bottles were amended with 0.8 mM of L-cysteine-HCl to reduce any remaining oxygen and the pH checked by removing an aliquot of brine. The needles were then removed and the serum bottles autoclaved at 121 °C for 20 minutes. Once cooled, 500 µl of the anoxic unsterile brine samples from the field were then injected into the brine media using the same set up. Soil inoculations were carried out by adding 1 g of soil to triplicate sterile serum bottles, filling them with sterile anaerobic media and sealing them as above inside the anaerobic chamber. Incubations were also carried out at 37 °C, but left for 60 days.



#### 4.1.2.2 Inoculating media with the brines

To further examine the habitability of the brines, a number of artificial media were created and inoculated with the brines. This involved both aerobic and anaerobic media. Initially only aerobic NaCl and KCl tryptic soy (Table 4.1a) and nutrient (Table 4.1b) broths were used to identify potentially uninhabitable brines (results on Table 4.4). This was done in tandem with the brine media enrichments in Section 4.1.2.1.1. A more diverse set of media were then used to interrogate the remaining brines.

Media enrichments used the same aerobic/anaerobic techniques as the brine media described above. Media recipes are shown in Tables 4.1 and Table 4.2. The media recipes were designed to provide a range of different carbon sources to meet the needs of a variety of organisms. The ionic profiles of the media were varied to mirror those found in the brines, but less extreme. For example, the HM media and the high NaCl media mirrored the high NaCl levels seen in 44 XC, Billingham and 215. The high  $\text{MgCl}_2$  Nutrient (Table 4.1d) and tryptic soy broth (Table 4.1c) both create a similar environment to 101-P. However, the  $\text{MgCl}_2$  levels were brought down to 2 M which is under the proposed  $\text{MgCl}_2$  limit to life (Hallsworth et al. 2007). The high KCl nutrient broth (Table 4.1f) and tryptic soy (Table 4.1e) broth media mirrors 29 XC. All media had a small amount of KCl included since it is commonly used as an osmoprotectant by extreme halophiles.

(a)

Chemical	Quantity
NaCl	250.0 g
KCl	2.0 g
Tryptic soy broth	25 g
dH <sub>2</sub> O	1000 ml

(b)

Chemical	Quantity
NaCl	250.0 g
KCl	2.0 g
Nutrient broth	19 g
dH <sub>2</sub> O	1000 ml

(c)

Chemical	Quantity
MgCl <sub>2</sub>	190.0 g
KCl	2.0 g
Tryptic soy broth	25 g
dH <sub>2</sub> O	1000 ml

(d)

Chemical	Quantity
MgCl <sub>2</sub>	190.0 g
KCl	2.0 g
Nutrient broth	19 g
dH <sub>2</sub> O	1000 ml

(e)

Chemical	Quantity
KCl	200.0 g
Tryptic soy broth	25 g
dH <sub>2</sub> O	1000 ml

(f)

Chemical	Quantity
KCl	200.0 g
Nutrient broth	19 g
dH <sub>2</sub> O	1000 ml

**Table 4.1.** Varieties of tryptic soy broth and nutrient broth (both Difco Laboratories, Detroit, Mich.) media recipes amended with different salts used to enrich organisms from the brines to examine habitability. The high MgCl<sub>2</sub> used (c and d) were balanced to pH 3.5 to closer match the pH in 101-P, the rest were pH balanced to 7 using sodium hydroxide and hydrochloric acid. The nutrient broth and tryptic soy broth were both made in house. The nutrient broth (b, d and f) consisted of D(+) glucose, peptone and yeast extract in final concentrations of 1 g/l, 15 g/l and 3 g/l respectively. The tryptic soy broth (a, c and e) consisted of casein peptone (pancreatic), soya peptone (papain digest) and dextrose at final concentrations of 17 g/l, 3 g/l and 2.5 g/l, respectively.

HM media is commonly used to enrich moderate halophiles from hypersaline environments and so was included as an additional media in case it provided something missing from the media detailed in Table 4.1 for any more fastidious organisms present in any of the brines. Due to the salinity of the brines, the NaCl concentration was increased from 178 g/l to 250 g/l.

Solution	Chemical	Quantity
1	NaCl	250.0 g
	MgSO <sub>4</sub>	1.0 g
	dH <sub>2</sub> O	500 ml
2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.36 g
	KCl	0.06 g
	NaBr	0.23 g
	FeCl <sub>3</sub>	Tr
	Protease-peptone	5.0 g
	Yeast Extract	10.0 g
	Glucose	24.0 g
	dH <sub>2</sub> O	500 ml

**Table 4.2.** HM media recipe from Vreeland and Hochstein (1992). The two solutions were mixed in a sterile Duran bottle in a flow hood after autoclaving. The pH of the two mixtures was balanced to 7.5.

#### 4.1.4 Water activity

Water activity is measured as the partial vapour pressure of water in a substance, divided by the partial vapour pressure of pure water at the same temperature. In a sealed container, the partial vapour pressure of water is the same as relative humidity. A water activity meter therefore measures  $a_w$  by measuring the relative humidity of the air above a solution inside a sealed container, and determining the ratio between it and the relative humidity of pure water at the same temperature (thus the relative humidity over a saturated NaCl solution is 75.5 compared to 100 over pure water giving an  $a_w$  of 0.755).

Water activity ( $a_w$ ) was measured in the laboratory using a Rotronic™ HP23-AW-A water activity meter. The water activity meter was calibrated using five points of reference, these being 0.935, 0.845, 0.755, 0.595, 0.325 composed of saturated calibration solutions of  $\text{KH}_2\text{PO}_4$ ,  $\text{KCl}$ ,  $\text{NaCl}$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{MgCl}_2$  respectively, as detailed in Winston and Bates (1960). Standards were made up in-house using 18.2 M  $\Omega$ -cm distilled water from a Thermo Scientific™ Barnstead™ NanoPure™ system and the relevant salts at saturation concentrations. To achieve saturation, the salts and water were left sealed in Duran bottles in a 30 °C warm room and mixed using magnetic stirrers for up to 24 hours until all the salts had dissolved.

Once calibrated, the sample cup was cleaned with distilled water, dried and filled with 5 ml of brine. It was then mounted on the water activity meter and sealed. Measurement took place by placing the entire water activity meter in a 30 °C incubator to ensure the temperature was kept consistent. Each brine was measured in triplicate.

The “AwE” mode on the Rotronic water activity meter was used to measure  $a_w$ . This method continuously measures temperature and humidity and waits for them to reach an equilibrium to determine relative humidity. Although this method is much slower than the “AwQuick” mode, which monitors humidity and temperature stability for a short period of time and then algorithmically extrapolates the equilibration point, it was found to produce more repeatable and reliable data when measuring standards.

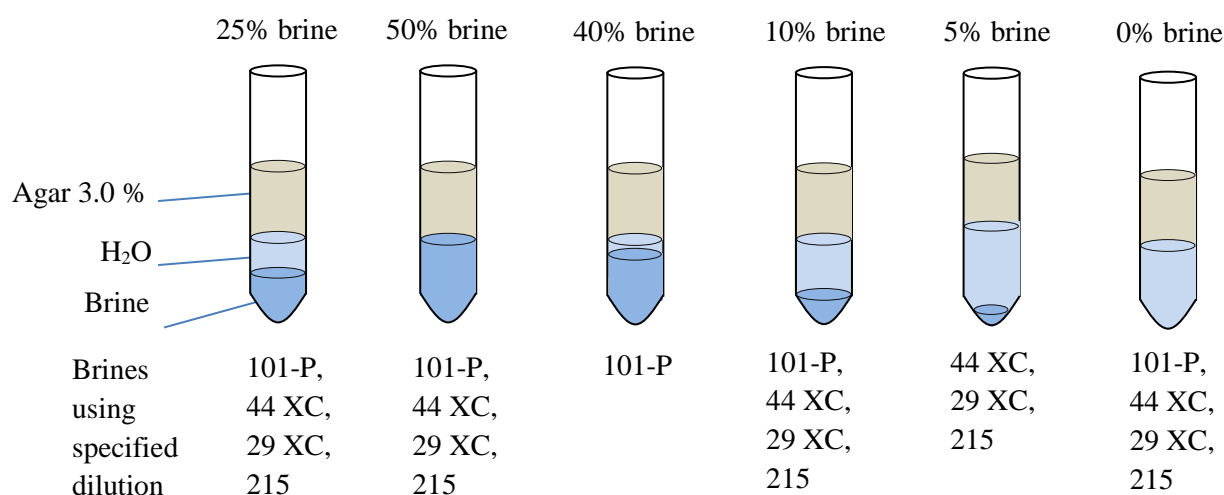
### **4.1.5 Chaotropicity and kosmotropicity**

Like water activity, determining the chao/kosmotropicity of a solution is useful for characterising the environmental stresses imposed on organisms within aqueous environments, particularly brines. The exact mechanisms behind chao/kosmotropicity are still not well understood making it difficult to accurately determine the chao/kosmotropicity of a solution given only its geochemical profile. Consequently, chao/kosmotropicity can only be quantified by observing the behaviour of macromolecular systems when interacting with the solution in question. In order to try and standardise the quantification of chao/kosmotropicity, a methodology for measuring it was developed by Cray et al (2013). This methodology uses the gel point of agar as a proxy for the chao/kosmotropicity of a substance. In this measurement system, a chaotropic substance is defined as a substance that when mixed with agar, depresses the agar’s gel point and a kosmotropic substance, a substance which increases its gel point. Whilst this assay is somewhat limited by only giving

the chao/kosmotropicity effect on one specific macromolecular system, it does provide an acceptable platform in which to compare between substances.

Four brines were investigated for chao/kosmotropicity in this study, these being 44 XC, 101-P, 29 XC and 215. Billingham Baths brine not assayed here due to its chemistry being essentially identical to brine 215 (see Section 3.3). All brine samples used were filtered through a 0.45  $\mu\text{m}$  and then 0.22  $\mu\text{m}$  filter to remove any insoluble material that might affect the spectrophotometer OD readings (see below).

As mentioned above, the effect of the brines on the gel point of agar was used as a proxy for chao/kosmotropicity. Agar was made up at a concentration of 3.0 % w/v using extra-pure reagent grade agar (Nacalai Tesque, Kyoto, Japan) and 18.2 M  $\Omega\text{-cm}$  distilled water from a Thermo Scientific™ Barnstead™ NanoPure™ system. This mixture was then heated using a microwave in a glass flask until the agar was fully molten. Dilutions of each brine were then set up in 15 ml centrifuge tubes and the agar added for final concentrations of (left to right on Figure 4.1) 50/25/25 (agar/water/brine), 50/50 (agar/brine), 50/10/40 (agar/water/brine), 50/40/10 (agar/water/brine) and 50/50 (agar/water). The final agar concentration in all six centrifuge tubes was therefore 1.5 % w/v as in Cray et al. (2013). A slightly different range of dilutions of each brine (see Figure 4.1, below each centrifuge tube) were used to ensure the best coverage of the agar/brine gelation curve which had been examined in some preliminary runs of the experiment. The tubes containing the brine/water mix were preheated to 80 °C in a water bath prior to the introduction of the agar to ensure it remained molten when mixed.



**Figure 4.1.** Schematic of agar/brine/water dilutions used to measure chao/kosmotropicity in the brines collected at Boubly. These were made up in 15 ml centrifuge tubes, mixed by inversion and 150  $\mu$ l aliquots were removed and placed in a 96 well plate. The brines using each specific dilution are listed under each schematic.

To accurately determine the exact moment in which agar has undergone gelification, a spectrophotometer is typically used. As agar solidifies, its optical density increases. The extra-pure reagent grade agar (Nacalai Tesque, Kyoto, Japan) used has a very well defined gel point of  $43.5 \pm 0.3$  °C (see Hallsworth et al. 2003). The OD at the point which the agar reaches this temperature can then be used to determine the gel point of multiple agar solutions containing chao/kosmotropic substances, provided the same concentration of agar was used for each. Although the methodology outlined in Cray et al. (2013) produces repeatable results for measuring chao/kosmotropicity, the fact that the agar must be carefully cooled in a water bath, and its OD measured by hand in a spectrophotometer, makes it very time consuming and inefficient. To improve on the efficiency of this methodology whilst retaining the quality of the data gathered, a Biotek Synergy 2 plate reader was used instead of a water bath and spectrophotometer.

This adapted methodology involved loading a 96 well plate including its lid into the plate reader and preheating it to 65 °C (the maximum temperature for a Synergy 2) for an hour to ensure the cooler plate did not solidify the agar whilst the plate was loaded. The agar/brine mixtures (150  $\mu$ l) were then pipetted into the wells on the plate in triplicate. During this procedure, the brine/agar solutions were kept in an 80 °C water bath to ensure the agar/brine mixtures remained molten. Those brines suspected of being chaotropic were dispensed first

into the 96 well plate. This is because chaotropic substances depress the agar gel point meaning they would be less likely to solidify whilst the other brine/agar mixtures were being dispensed. Once this was complete, the lid was placed on the 96 well plate and it was returned to the plate reader.

The plate reader was programmed to hold the temperature at 65 °C for an hour to ensure a uniform temperature between the wells and lid to prevent condensation. The programme then decreased the temperature 1 °C every 15 minutes and measured the OD of the agar brine mixture in each well at 500 nm. This continued until the plate reached room temperature.

It is worth noting that had a brine been extremely chaotropic, the agar gelation point could have been below room temperature. Since the biotek plate reader cannot cool below room temperature, the agar would have remained molten. Similarly, had it been extremely kosmotropic, it would have solidified the agar upon mixing at 80 °C. For these reasons, several preliminary runs of the experiment were undertaken to ensure brine dilutions were adequate to allow gelation or prevent immediate gelation during mixing. Using a plate reader with hardware able to lower the temperature below room temperature would make this unnecessary for chaotropic agents.

To determine the change in gel point, the optical density of the 50/50 agar/water mixture (1.5 % agar with no brine), was measured at 43.5 °C over the three triplicates and the mean taken. Because the plate reader could only be set in 1 °C increments and the gel point of the agar was known to be 43.5 °C, the OD readings between 44 and 43 °C were plotted on a line graph and the OD value at 43.5 °C calculated from the linear best-fit line. The temperature where the OD of the brine/agar samples, matched the agar/water OD at 43.5 °C, were then assumed to be the point in which gelification occurred in the brine/agar mixtures. These gelation temperatures for the different concentrations of brine in the agar mixtures (i.e. 50/50 agar/brine, 50/25/25 agar/water/brine etc), were then plotted on line graphs and the temperature at which a 100 % brine concentration would have caused gelation determined. From this, the heat capacity was calculated to compare between substances (expressed in kJ kg<sup>-1</sup>).

Chaotropic substances increase a liquid's heat capacity and a kosmotropic substance decreases it. Heat capacity is directly related to gelification point, since a substance with a higher heat capacity retains more energy at a lower temperature. Molten agar requires a decrease in heat energy to undergo gelification and consequently, a chaotropic substance which raises the heat capacity of the agar, also decreases the temperature under which it

undergoes gelification. As mentioned however, chao/kosmotropicity is a black box term and therefore does not explicitly describe the mechanism behind this change, only its effects.

### 4.1.6 Ionic strength

Despite being known as having an impact on the function and structure of biological macromolecules (e.g. Baldwin 1996, Kunz et al. 2004) ionic strength has only recently been identified as a potential physicochemical limiter on the habitability of specific aqueous environments (Fox-Powell et al. 2016). Ionic strength is a unit indicating the strength of the electric charge in a solution, determined by multiplying the ion concentration by the square of the ion's charge (Equation 4.1). Ionic strength is generally higher the more positively and negatively charged multivariate ions in a solution.

**Equation 4.1** 
$$I = 0.5 \sum c_i z_i^2$$

$c_i$  = concentration of ion i (in mol litre<sup>-1</sup>)

$z_i$  = charge of ion i

This value is simply calculated from the ion data collected using ICP and IEC. It is worth noting that this data set would only be complete had we assayed for every potential ion in existence. Since that is not possible, the ionic strength values presented here can be considered a lower limit.

## 4.2 Results

### 4.2.1 Enrichment results

Enrichment work to examine habitability in the brines produced a diversity of results.

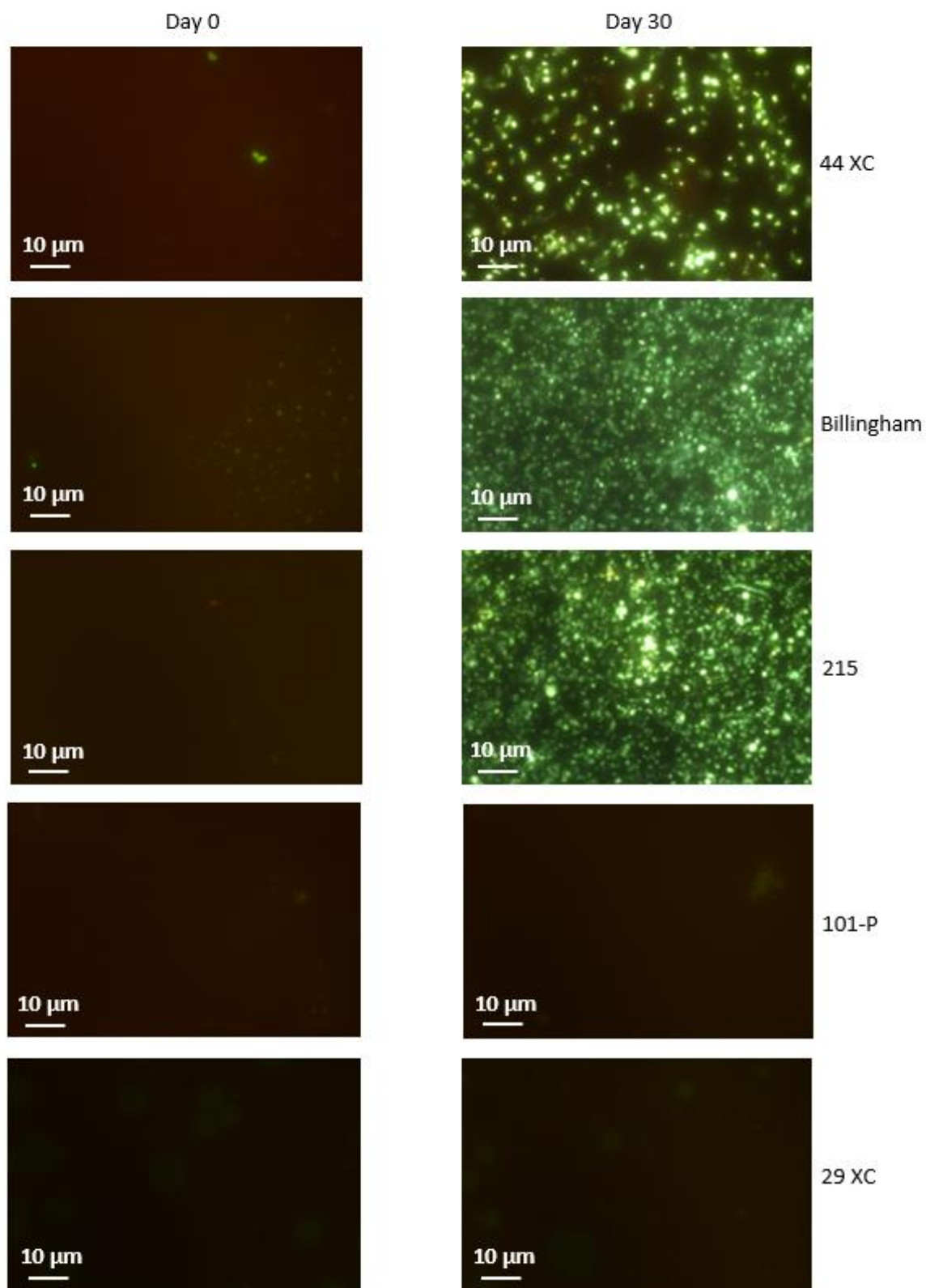


#### 4.2.1.1 Initial inoculation of brines into the same brine with added carbon source

Using the unsterilised environmental samples of 215, 44 XC and Billingham brines to inoculate enrichment media composed of the same brine with addition of a carbon source (e.g. sterilised 215 brine supplemented with a carbon source was inoculated with unsterilized 215) (see Section 4.1.2.1) resulted in rapid growth in all three brines in every replicate. In contrast, neither 101-P, nor 29 XC produced any visible growth (see Table 4.3). Microscopy images of these brine cultures are shown in Figure 4.2.

		+/- growth in the brines for each triplicate				
Inoculant	Carbon Source	215	44 XC	Billingham	29 XC	101-P
Unsterilised brine	Yeast	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
Unsterilised brine	Na pyruvate and casamino acids	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-

**Table 4.3.** Results of attempts at enriching organisms in the brines to test habitability after 30 days of enrichment at 37 °C. Each condition was done in triplicate (- no growth, + growth). This experiment amended filter sterilised brines using two sets of carbon sources (yeast extract and Na pyruvate and casamino acids) and used the same brines, but unsterilised, to inoculate the carbon amended brines. 215, 44 XC and Billingham are all clearly habitable and rapidly became opaque with high densities of halophiles. 29 XC and 101-P both failed to produce any kind of microbial enrichment.



**Figure 4.2.** Microscopy of cultures in filtered brines containing nutrient broth inoculated with the same brines unsterilised. 44 XC, Billingham and 215 all displayed dense enrichments after less than 30 days. 29 XC and 101-P showed no growth after 30 days.

#### 4.2.1.2 Initial inoculation of brines into hypersaline media

Using the brines to inoculate a narrow range of aerobic enrichment media (methods in Section 4.1.2.2) also produced similar results to Section 4.2.1.1. 215, 44 XC and Billingham all produced growth in the three media initially tested. Neither 29 XC nor 101-P produced any visible microbial growth.

	Inoculum				
Media	215	44 XC	Billingham	29 XC	101-P
High NaCl + KCl nutrient broth	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
High NaCl + KCl tryptic soy broth	+/+/+	+/+/-	+/+/+	-/-/-	-/-/-

**Table 4.4.** Results of initial attempts at enriching organisms in two media types to test habitability after 30 days of enrichment at 37 °C. Each condition was done in triplicate (- no growth, + growth). 215, 44 XC and Billingham consistently produced growth when used as an inoculum in these two media types. Both 29 XC and 101-P failed to produce microbial growth. One of the 44 XC triplicates did not produce any growth.

#### 4.2.1.3 Expanded inoculation of brines into hypersaline media

The data above showed that that Billingham, 215 and 44 XC were capable of supporting microbial growth and could therefore be defined as habitable. 29 XC and 101-P appeared at this point to be uninhabitable. To further test this, an expanded set of media was used in an attempt to culture organisms from brines 29 XC and 101-P to increase the chances of a positive enrichment (Table 4.5). The results showed that neither brine could be used to start an enrichment culture.

	Inoculum	
Media	29 XC	101-P
Aerobic high MgCl nutrient broth	-/-/-	-/-/-
Aerobic high MgCl tryptic soy broth	-/-/-	-/-/-
Aerobic HM Media	-/-/-	-/-/-
Aerobic high KCl nutrient broth	-/-/-	-/-/-
Aerobic high KCl tryptic soy broth	-/-/-	-/-/-
Anaerobic high MgCl nutrient broth	-/-/-	-/-/-
Anaerobic high MgCl tryptic soy broth	-/-/-	-/-/-
Anaerobic HM Media	-/-/-	-/-/-
Anaerobic high KCl nutrient broth	-/-/-	-/-/-
Anaerobic high KCl tryptic soy broth	-/-/-	-/-/-

**Table 4.5.** Results from expanded set of culturing using brines 29 XC and 101-P as inoculum (see Table 4.1 for recipes). Each condition was done in triplicate (- no growth, + growth). Neither brine was able to produce a single positive enrichment across the media tested. Anaerobic cultures were grown for 60 days and aerobic cultures 30 days, both at 37 °C.

#### **4.2.1.4 Expanded inoculation of brines with added carbon source**

In addition to this more diverse set of media, a greater range of inoculum were added to carbon enriched versions of brines 29 XC and 101-P (Table 4.6) to increase the chances of a positive enrichment. This experiment was designed to examine whether either brine was habitable, but contained only dead or inactive organisms. A number of the 101-P brines inoculated with soil were transferred after 30 days to a fresh filter sterilised 101-P containing yeast extract or Na pyruvate and casamino acids. This was to remove insoluble matter and allow better examination of any organism growing in the brine.

Inoculant	Carbon Source	+/- growth in the brines for each triplicate	
		29 XC	101-P
215	Yeast	+/+/+	-/-/-
215	Na pyruvate and casamino acids	+/+/+	-/-/-
44 XC	Yeast	+/+/+	-/-/-
44 XC	Na pyruvate and casamino acids	+/+/+	-/-/-
Billingham	Yeast	+/+/+	-/-/-
Billingham	Na pyruvate and casamino acids	+/+/+	-/-/-
215, 44 XC, Billingham, yeast media enrichment cocktail	Yeast	+/+/+	-/-/-
215, 44 XC, Billingham, yeast media enrichment cocktail	Na pyruvate and casamino acids	+/+/+	-/-/-
Soil	Yeast	n/a	-/-/-
Soil	Na pyruvate and casamino acids	n/a	-/-/-
Billingham, 44 XC, 215 cocktail (anaerobic)	Yeast	n/a	-/-/-
Soil (anaerobic)	Yeast	n/a	-/-/-

**Table 4.6.** Results from broader range of enrichments carried out in brines 29 XC and 101-P (filter sterilised with added carbon sources). Each condition was done in triplicate (- no growth, + growth). Soil and anaerobic enrichments were carried out as additions on 101-P to broaden the chances of a positive enrichment. Anaerobic cultures were grown for 60 days and aerobic cultures 30 days, both at 37 °C. The cocktail inoculum was taken from positive 215, 44 XC, Billingham yeast media enrichments.

All triplicates failed to produce growth in 101-P, but succeeded in 29 XC. Despite the lack of anything growing when using 29 XC as an inoculum, growth was possible in the brine when amended with carbon sources and inoculated with some of the other brines collected, indicating the brine is habitable. 101-P failed to produce any positive enrichments under any experiment attempted.

### 4.2.2 Water activity

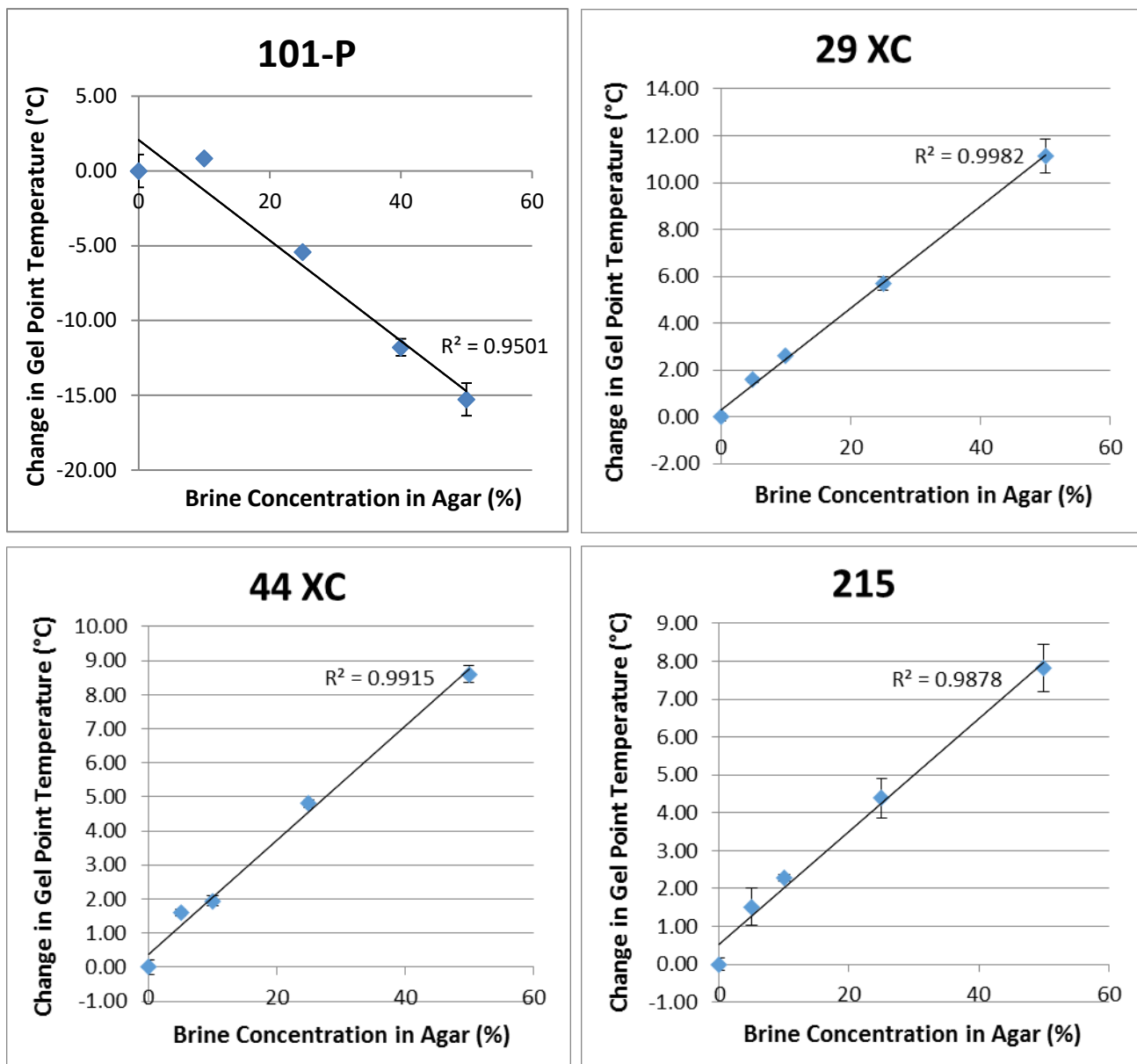
The water activity observed in the brines was relatively consistent amongst four of the brines. Billingham Baths, 215, 29 XC, 44 XC all ranged from 0.72 to 0.742, despite 29 XC displaying quite a different elemental profile. 101-P is significantly lower than the other brines at 0.566.

	$a_w$
<b>Billingham Baths</b>	0.727
<b>215</b>	0.720
<b>29 XC</b>	0.730
<b>44 XC</b>	0.742
<b>101-P</b>	0.566

**Table 4.7.** The table above shows the different water activity values recorded in each brine collect from the mine at 30 °C.

### 4.2.3 Chaotropicity/kosmotropicity assay

The brines had a significant effect on the agar gel points even at low concentrations. The majority of the brines increased the temperature in which this gel point occurred. Brine 101-P however, decreased it (see Figure 4.3).



**Figure 4.3.** Change in gel point temperature plotted against brine concentration in agar. The addition of brines altered the agar gelation temperature significantly in the four brines tested. 29 XC, 44 XC and 215 are shown to increase the gel point temperature of the brines, whilst 101-P decreases it significantly. Triplicate values represent experimental rather than measurement triplicates.  $R^2$  values are consistently above 95.

The graphs in Figure 4.3 were used to determine the change in gel point temperature at 100 % brine concentration. This was then multiplied by the known heat capacity of the agar-water system (4.14 kJ kg) to get the full brine heat capacity (see Table 4.8) (Cray et al., 2013).



	<b>44 XC</b>	<b>215</b>	<b>29 XC</b>	<b>101-P</b>
<b>Extrapolated change in gel point temperature at 100% brine concentration</b>	17.10	15.44	22.06	-31.24
<b>Change in heat capacity (kJ kg<sup>-1</sup>)</b>	-70.98	-64.08	-91.56	129.63

**Table 4.8.** Change in gel point temperature caused by the brines. From these values, the change in heat capacity was then calculated.

From the data in Table 4.8, it is clear that the majority of the brines decrease the heat capacity of the agar. 44 XC and 215 display a very similar effect on the temperature at which the agar/brine solutions underwent gelation, with heat capacities changes of  $-70.98 \text{ kJ kg}^{-1}$  and  $-64.08 \text{ kJ kg}^{-1}$ , respectively. This behaviour is consistent with their similar ionic profiles (see Table 3.3). 29 XC is slightly more kosmotropic, with a heat capacity change of  $91.56 \text{ kJ kg}^{-1}$ . 101-P is distinctly chaotropic, significantly depressing the gelation point of the agar and increasing heat capacity  $+129.63 \text{ kJ kg}^{-1}$ .

#### 4.2.4 Ionic strength

Ionic strength values were calculated from  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  concentrations in the brines. The values found in the brines are relatively similar, between 5.32 to 6.56 mol/l (Table 4.9). 101-P was again the most extreme brine, with the highest ionic strength of 6.56 mol/l.

	Ionic strength (mol/l)
<b>Billingham</b>	5.32
<b>215</b>	5.38
<b>44 XC</b>	5.55
<b>29 XC</b>	5.32
<b>101-P</b>	6.56

**Table 4.9.** Ionic strength for the brines collected during this survey calculated from ions assayed in Chapter 3. Values are fairly consistent between the brines, although are slightly higher in 101-P.

## 4.3 Discussion

### 4.3.1 Brine habitability

The culturing experiments clearly show that habitability varies within the brines. It is worth noting that it would always be possible broaden the range of carbon sources, growth temperatures, inoculants and ionic compositions of the media to increase the chances of a positive enrichment. Additionally, many microorganisms on Earth are not culturable with modern enrichment techniques. This was in some way mitigated during these experiments by using communities of organisms from a number of different sources as opposed to isolates to better simulate the complexity of natural environments. Within the confines of practicality, the combinations of enrichment techniques used here should be viewed as estimates, not definitive tests of habitability.

Organisms grew successfully in 44 XC, Billingham and 215 when amended with carbon sources. Communities within those brines were also able to start enrichment cultures in every media condition tested. The geochemical profiles of these brines are similar to solar

salterns, with NaCl being the dominant ion present, so their habitability was expected. These communities are investigated in more detail in Chapter 5.

In contrast, brine 101-P appears to be uninhabitable (within the limitations of the experimental design). All enrichments attempted within it failed to produce growth. It also failed to start any of the enrichment cultures tested. Both results suggest the brine is uninhabitable and does not contain viable organisms. The physicochemical reasons for the brine's inability to support biological activity are examined below in Section 4.3.2.

Brine 29 XC is more complex. No organisms could be cultured from the brine. However, enrichment cultures within the brine were successful when using other brines as an inoculum, but failed when using 29 XC itself. This suggests the brine is habitable, but does not contain viable organisms. The reason for this could be attributed to two things. The brine was highly pressurised at the sampling point. In order to sample it, the brine had to undergo rapid depressurisation. This may have killed the organisms present within the brine. Alternatively, or additionally, the combined stressors of high pressure and high KCl concentrations, may have severely limited microbial propagation prior to depressurisation. This latter explanation is supported by the fact that DNA could not be extracted from 29 XC (see Chapter 5) which should have been possible if the organisms had simply died due to depressurisation. However, there may have been very low concentrations of viable organisms present before depressurisation, but were too low in abundance to show up in the DNA extractions. A further reason could be that the brine only contains organisms that cannot grow using the enrichment methods employed in this study. It is still possible however, that pressure plays a role in determining habitability in the Zechstein sequence. Few studies have looked at polyextremophiles (Harrison et al. 2013) and no studies appear to have examined the ability of organisms to grow at the KCl concentrations present in 29 XC under the high pressure recorded. This makes it hard to judge the additional stress imposed by raised pressure in this type of environment, although it may have contributed to the lack of organisms present in 29 XC.

### **4.3.2 Extremes in the brines**

A number of physicochemical characteristics and the interactions between them define the habitability of aqueous environments. As discussed, certain extremes alone can limit habitability where all other physicochemical characteristics are benign.

The chao/kosmotropicity recorded here appear to be permissive to life in the majority of brines, aside from 101-P. Brines 44 XC, 215 and 29 XC are all kosmotropic. The more kosmotropic behaviour seen in 29 XC can be attributed to it being dominated by KCl. In comparison, brine 44 XC and 215 are dominated by NaCl, which is slightly less kosmotropic than KCl (Cray et al. 2013). Unlike chaotropicity, there is no a currently defined upper habitability limit relating to kosmotropicity, and it may not explicitly act as a barrier to life without additional stressors. Brines 44 XC, 215 and 29 XC are therefore habitable when considered with this physicochemical proxy. Since Billingham Baths brine is chemically very similar to 215, it is also assumed to display a similar kosmotropicity.

Although brines 44 XC, 215, 29 XC and Billingham Baths all have fairly low water activity, they lie comfortably above the currently defined water activity habitability limit of around 0.650–0.605 (Stevenson et al. 2015a and b). Temperature, pH, and the lack of high concentrations of heavy metals such as iron, also support these brines being habitable within currently defined limits which is consistent with the results of the enrichment work. The additional stress imposed by pressure in 29 XC could have made the fluid uninhabitable, despite the unpressured brine clearly being habitable. This is discussed further at the end of Chapter 5.

Brine 101-P's high concentration of both Mg and Cl combined is likely to be problematic for life.  $\text{MgCl}_2$  is a well-known chaotropic substance, responsible for rendering certain deep-sea basins uninhabitable (see Hallsworth et al. 2007, Yakimov et al. 2015). Attempts have been made to define a chaotropic barrier to life using these deep-sea environments. This was suggested by Hallsworth et al. (2007), to be at a chaotropicity equivalent to 2.3 M of  $\text{MgCl}_2$ , when other competing kosmotropes were not present.  $\text{MgCl}_2$  raises a solutions heat capacity by 54 kJ kg<sup>-1</sup> per mole, equivalent to 124.2 kJ kg<sup>-1</sup> at 2.3 M. In comparison, brine 101-P increases heat capacity to 129.63 kJ kg<sup>-1</sup>. This indicates that brine 101-P does not contain substantial amounts of kosmotropic substances such as sulphates that are powerful enough to counteract this concentration of  $\text{MgCl}_2$ , meaning the brine displays a chaotropicity beyond the current defined limit for life. Again, this is consistent with the enrichment results.

101-P is also extreme when considered with respect to water activity. At 0.566, it is below the currently defined water activity limit (Stevenson et al. 2015). It also has a low pH of 3.42, which independently is not a barrier to life, but could act in concert with other the other stressors to add to the hostility of the brine to microorganisms (Harrison et al. 2013).

Ionic strength does not appear to be a barrier to habitability in any of the brines collected during this study. The proposed limit for ionic strength in the brines was shown by Fox-

Powell et al. (2016), to be around 12 mol/l, depending on other factors such as water activity. Ionic strength does not appear to be a primary factor in the brines collected in this study considering the more extreme water activity and chaotropicity measurements recorded.

### **4.3.3 Brines habitability and its link to brine origins**

Chapter 3 used isotope, major elements and ion geochemistry to determine where the brines originated in the evaporite sequence. With this information, it is possible to discuss the links between habitability and brine origin and evolution in the Zechstein sequence.

All the brines demonstrated to be habitable originated from the Sherwood Sandstone aquifer. The only brine that appeared to be uninhabitable (101-P), originated partly or wholly from an unidentified separate source. Mg and Cl rich brines typically display low water activities and strongly chaotropic behaviours (Hallsworth et al. 2007) meaning the high concentration of Mg present within 101-P appears to be the reason for it being uninhabitable when the other Cl rich brines are habitable.

As discussed in Chapter 3, this Mg was probably not present in the original water of crystallization fluid, but was later acquired through interactions with the thick dolomitised limestone beneath the mine. Mg-rich dolomitised limestone is commonly associated with evaporite deposits and forms when calcium ions in calcite are replaced with magnesium and recrystallized to dolomite. This process is common in sabkha regions and dolomitised sedimentary rocks are frequently found in evaporite deposits, but can also form through a number of different diagenetic processes (Kinsman 1973, Machel and Mountjoy 1986, Machel et al. 2004). Thick regions of dolomite do not exist on the upper layers of the mine meaning that the fluids moving down from the Sherwood Sandstone tend not to have such high Mg concentrations. Some of the other brines do display moderate Mg enrichment such as 29 XC and 44 XC, which probably results from their exposure to the thinner dolomitised shale horizon of the Upgang Formation.

It is therefore possible to suggest that these dolomitised regions are very important for determining the habitability of fluids within the Zechstein sequence. Other evaporite minerals such as carnallite (not thought to be related to 101-P) could also have impacts on fluid habitability in this sequence (e.g. Hallsworth et al. 2007). These processes demonstrate that despite existing 250 million years ago, ancient environmental conditions can still have tremendous impacts on the habitability of modern deep subsurface environments.

## 4.4 Conclusion

The aim of this chapter was to examine the habitability of the brines collected from Boulby Potash mine using enrichment work and measurements of the brine's physicochemical properties. The following conclusions can be drawn from this work:

- The majority of the brines collected were habitable despite some variety in their geochemical composition.
- Within the limitations of the methods used, one brine, 101-P, appears to be uninhabitable based on enrichment culturing and physicochemical proxy measurements. The brine's low water activity and chaotropic properties seem to responsible for this lack of habitability.
- Ionic strength is benign in all the brines and unlike certain Martian environments, appears to have little impact on the habitability of deep subsurface brines in the Zechstein.
- All the habitable brines appear to have originated from the Sherwood Sandstone aquifer. The uninhabitable brine was from an unknown source, but likely interacted with dolomite.
- The remains of a 250-million-year old ancient sea still strongly governs the habitability of this modern day deep subsurface environment. This makes the origin and evolution of the brines within the Zechstein important controls on fluid habitability. Contact with Mg rich dolomitic layers, for example, can render a fluid uninhabitable.
- Fluid habitability can change extremely rapidly over small scales within the Zechstein deposit. Most of the brines were sampled within a few km's of each other at similar depths, but vary in their ability to support biology.
- Pressure may also play an important role in determining habitability within deep subsurface evaporite deposits, as may be the case in brine 29 XC.

Overall this work has shown that both habitable and uninhabitable fluids exist within the Zechstein sequence. This work could be greatly expanded in the future. More brines could be sampled in the Zechstein sequence and other evaporite deposits around the world to see how habitability changes over a larger dataset and on a regional scale. The range of enrichment

conditions could be greatly expanded when examining habitability in brines such as 101-P. For example, a range of low salt media could be used to try and culture organisms. Enrichment work could also take place in brines like 29 XC whilst under pressure, to see how this influences their habitability. Culturing at low water activity levels, high chaotropicity and ionic strength could all lead to the isolation of important organisms from these types of brines. Further study of life in these environments has the potential to elucidate new methods of microbial survival in extremes, redefine the boundaries of habitability and expand our search for life beyond Earth to new environments.

## Chapter 5: The taxonomic structure and functional capabilities of hypersaline deep subsurface brines

As discussed in Chapter 2, deep subsurface evaporite brines are both poorly studied and important environments for understanding the habitability of the terrestrial and Martian subsurface. Despite their importance, work examining the full metagenomic profile of these brines is extremely limited, meaning little is known about how community structure changes across brines with varying compositions and/or formational histories. The different brines collected from Boulby during this study, characterised in Chapter 3, allowed us to start filling this knowledge gap by investigating the microbial communities present in a range of different brine compositions, each with varying origins and evolutionary histories. To characterise the organisms present in each brine and improve understanding of how they survive in the deep subsurface, the following questions were asked:

- i. What types of organisms are present in the brines and what functional pathways are present? These are the most basic questions, and will determine the types of organism and metabolic pathways present in the brine seeps, giving some idea of the methods used by life to survive in these deep subsurface environments.
- ii. How do the taxonomic and functional profiles of the deep subsurface brines compare to communities in surface hypersaline environments? Solar salterns are well characterised hypersaline habitats. The taxonomic and functional profiles generated here will allow us to compare community structure between comparable surface and deep subsurface environments.
- iii. Do the geochemical profile and/or origins of the brines have an impact on the microbial population? The influence of the different migration routes taken by the brines and therefore the different ions they contain, as discussed in Chapter 3, may influence the microbial population present.
- iv. Do any brine seeps not contain extractable DNA? If so, how does this relate to the physicochemical properties of the brines? Through the measurement of various chemical proxies such as water activity and culturing experiments, certain brines were predicted to be uninhabitable in Chapter 4. These conclusions can also be tested by examining the extractable DNA across the brines.



To answer these questions, DNA extractions were attempted on all the brines collected using a range of methods. Following successful DNA extraction from brines 215, 44 XC and Billingham Baths, metagenomic sequencing was carried out using Illumina sequencing technology. Using these data, functional and taxonomic profiles of the brines were generated, examined and compared to surface examples.

Due to the difficulties encountered with extracting DNA from the samples, some focus of the chapter is given to the different DNA extraction methods attempted. This should help future workers avoid the same issues experienced here when trying to extract DNA from Boulby and other similar environments.

## **5.1 Materials and methods**

### **5.1.1 Sampling**

Brines were sampled from the identical field sites at the same times as outlined in Chapter 3. Billingham baths and 215 were collected in January 2014 and the rest of the brines in February 2015. The samples collected for DNA extractions were all treated the same way. Brine was collected from the pools (Billingham Baths, 215 and 101-P) using sterile 50 ml centrifuge tubes. Seeps (44 XC and 29 XC) were collected at taps (as described in Chapter 3) installed by the mine to control brine flow, again in sterile 50 ml centrifuge tubes. All the centrifuge tubes were immediately placed in a cooler box with ice packs after collection and transported to the surface of the mine. Here, the brines were filtered using MO BIO 0.22  $\mu\text{m}$  water filters attached to a peristaltic pump inside a laminar flow hood. The filters were then rolled up using a pair of flame sterilised tweezers and placed in fresh sterile centrifuge tubes. The tubes containing the filters were then packed away into coolers with fresh ice packs added and transported back to the University of Edinburgh where they were immediately frozen at  $-20\text{ }^{\circ}\text{C}$ . The samples were therefore in a cooled state ( $\sim 4\text{ }^{\circ}\text{C}$ ) for around 5 hours between initial sampling and freezing. It was not possible to freeze the samples any sooner due to difficulties involved with transporting dry ice or a portable freezer into the mine. However, given the slow growth rates of communities in hypersaline environments and the low temperatures, changes to the microbial community would have been minimal during this time period. Salt rocks were collected from recently fallen fresh material in sterile bags with

sterile gloves and briefly flamed on the exterior in the lab to minimise external contamination.

Blank samples were also collected during these sampling trips. These consisted of simply pouring molecular grade H<sub>2</sub>O into a centrifuge tube in the mine. The blanks then underwent all the filtering, DNA extraction, concentration, PCR and sequencing processes that the rest of the samples did to control for contamination.

### **5.1.2 DNA extraction**

DNA extraction from environmental samples can be challenging. A range of techniques were examined in order to find a successful method for extracting DNA from the brines collected. Previous work on similar environments have employed a range of methods, including phenol-chloroform (e.g. Radax et al. 2001) and various extraction kits (e.g. Gramain et al. 2011, Park et al. 2009). Initial difficulties with extracting DNA from Boulby during this study led to a number of these methods being attempted with mixed results. Only brines 215 and Billingham had the full range of DNA extraction methodologies tested on them due to the amount of material available.

As mentioned above, brines were firstly filtered using MO BIO 0.22 µm water filters attached to a peristaltic pump inside a laminar flow hood. Approximately 10 L of each brine was filtered. These filters were then removed from their plastic casing with flame sterilised tweezers, placed in a 50 ml centrifuge tube and stored at -20 °C. Due to the clays present and the liquid density, this required up to 5 filters per L of fluid in brine 101-P. DNA extraction was then performed on these filters. For the solid salt samples, the larger samples were broken up into smaller (~10 cm wide) pieces inside a flow hood and then passed under a flame briefly to sterilise the outside. They were then placed in 50 ml centrifuge tubes (~10 g per tube) for storage.

A phenol-chloroform method was initially tried using the methodology outlined below from Urakawa et al. (2010). Two filters from 215 and Billingham were cut into fragments using flame sterilised scissors in a flow hood and placed into a 2 ml Eppendorf tube with 250 µl of 2 x TENS buffer. The Eppendorf tubes were then incubated at 80 °C for 40 minutes. During this incubation, the tubes were agitated and the pressure relieved every 15 minutes. Following incubation, 1x volume of Tris saturated phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio at pH 8.0 was added to each tube and mixed with the pipette gun tip. The tubes

were then centrifuged at 16,000 g for 5 minutes, after which the supernatant (aqueous phase) was transferred to a fresh sterile 2 ml Eppendorf tube. The phenol/chloroform/isoamyl alcohol and centrifugation steps were then repeated to clean the DNA extraction further. The resulting supernatant was transferred to a new sterile 2 ml Eppendorf tube containing 430 µl of 7.5 M ammonium acetate and mixed by inversion. An equal volume (as the combined volume of supernatant and ammonium acetate) of chloroform was then aliquoted into the tube, mixed by inversion and centrifuged at 16,000 g for 5 minutes. The supernatant was transferred into new 2.0 ml centrifuge tube containing ice cold isopropanol (0.6x volume) and mixed by inversion. These new tubes were placed in -80 °C freezer for 10 minutes, and centrifuged again at 16,000 g for 30 minutes. The supernatant was again removed without disturbing the pellets, which were then washed with cold 80 % ethanol, centrifuged at 16,000 g for 30 minutes and air dried in the flow hood. Once all the ethanol had evaporated the pellets were re-suspended in 50 µl TE buffer.

Two kits were also used to attempt to extract DNA. A FastDNA® SPIN Kit and a MO BIO PowerMax® Soil DNA Isolation Kit. For both kits, the standard protocol supplied with the kits was used to extract DNA.

In addition to these techniques, two modifications were used in attempt to recover DNA from the samples. Liquid nitrogen grinding was attempted in case cell walls of the halophiles were proving resistant to the lysis buffer. This involved taking the brine in a tube, wrapping it in a wire and dipping it into liquid nitrogen until the bubbling subsided (i.e. it had become sufficiently cold). A flame sterilised steel rod was then used to mechanically grind the frozen brine. This proved difficult due to the high salinity causing rapid melting. DNA extractions using the phenol-chloroform and DNA kits were then used as described above to attempt to produce amplifiable DNA.

Geochemistry work showed that a significant portion of illite was present in the halite, sylvinite and brines analysed with XRD (see Section 3.3.2). Clays have been shown to interact with DNA extractions in the past (e.g. Cai et al. 2006), via the activate sites bonding to free DNA post lysis. This means that during centrifugation, instead of the DNA remaining in suspension, it is forced to the bottom of the column and discarded with the clays and other detritus. Methods have been developed to help deal with this issue in other extreme clay rich environments. It was therefore decided to include a phosphate buffer step into the MO BIO PowerMax® Soil DNA Isolation Kit protocol, derived for the work of Direito *et al.* (2011).

To achieve this, a 1 M phosphate buffer at pH 8 was made up in a 500 ml Duran® bottle. This contained 4.71 g of monosodium phosphate monohydrate, 124.85 g of disodium phosphate heptahydrate, 75 ml of molecular grade ethanol, top up to 500 ml with molecular grade H<sub>2</sub>O. The buffer was heated to 50 °C in a water bath to help dissolve all the components. Once dissolved, the phosphate buffer was filter sterilised with a syringe and 0.22 µm filter into a pre-sterilised 500 ml Duran® bottle inside a laminar flow hood. After filtration, the buffer was ready to include in the kit protocol. The water filters were firstly placed into a bead beating tube. In the case of the halite and sylvite, 10 g of material was added to the bead beating tubes. 40 ml of phosphate buffer was then added to the bead beating tube with 1.2 ml of lysis buffer (Solution C1 in the kit). These tubes were then fixed to a MO BIO vortex and shaken at the highest speed for 10 minutes. After vortexing, the tubes were placed in a water bath at 80 °C for 40 minutes and the pressure released twice during this period. The protocol then picked up at step 5 in the standard kit protocol.

In a number of the brines where DNA concentrations were exceptionally low, a custom DNA concentration method adapted from Urakawa et al. (2010), was used to help combine and concentrate the products of the extractions. This involved taking 250 µl of DNA extraction from the MO BIO kit and placing it in a DNA/RNA free sterile 1ml Eppendorf tube. Following this, 215 µl of 7.5 M ammonium acetate solution was added and mixed by inversion. To help visualise the DNA pellet and enhance recovery, 4 µl of linear acrylamide was added. Linear acrylamide has been used to help precipitate picogram amounts of DNA (Gaillard and Strauss 1990). Ice cold molecular grade isopropanol (240 µl) was then added, the tubes incubated at -80°C for 10 minutes and centrifuged at 16,000 g for 30 minutes. After centrifugation, the supernatant was removed and the pellet washed with 80 % molecular grade ethanol and centrifuged again at 16,000 g for 30 minutes. Once the pellet was air dried, it was re-suspended in 50 µl of TE buffer.

DNA extraction quality was checked throughout using two techniques a NanoDrop Lite and PCR amplification. Due to the salinity of the environmental samples, both bacteria and archaea specific PCR was carried out on all the extractions. DNA from *E. coli* and *Haloferax volcanii* were used as positive controls for bacteria and archaea respectively, in the DNA extraction and the PCR reaction, as well as molecular grade water for negative controls. PCR was carried out using the following reagents in RNase-, DNase-, DNA- and pyrogen-free 0.2 ml PCR tubes:

<i>GoTaq</i> ® Master Mix	12.5ul
Forward Primer	1 µl
Reverse Primer	1 µl
DNA sample	1 µl
Molecular grade water	9.5 µl

Primers used for bacteria specific PCR were 27F and 1389R, and for the archaeal specific PCR, 21F and 958R.

Primer	Sequence	Specificity
<b>27F</b>	AGAGTTTGATCMTGGCTCAG	Bacteria 16S rRNA
<b>1389R</b>	ACGGGCGGTGTGTACAAG	Bacteria 16S rRNA
<b>21F</b>	TCCGGTTGATCCYGCCGG	Archaea 16S rRNA
<b>958R</b>	GGACTACVSGGGTATCTAAT	Archaea 16S rRNA

**Table 5.1.** Detail on primers used for PCR reactions.

Two different thermocycling programmes were used to carry out the PCR reactions, optimised for the primers used. These are shown below:

**Bacteria PCR Conditions (27F/1389R)**

94°C	2 minutes	
94°C	1 minute	} Repeat cycle 25x
55°C	1 minute	
72°C	1:30 minute	
72°C	10 minutes	

## Archaeal PCR Conditions (21F/958R)

98°C	1 minute	
92°C	45 seconds	} Repeat cycle 35x
45°C	45 seconds	
72°C	45 seconds	
72°C	5 minutes	

After thermocycling, the PCR products were run through an electrophoresis gel to determine amplification success. A 1 % agarose gel (1 g agarose per 100 ml of TBE buffer) was made up in a glass conical flask, melted in a microwave, and left to cool. Once cooled slightly, 10 µl of SYBR® Safe DNA gel stain per 100 ml of gel was aliquoted into the conical flask and mixed gently. The molten gel was then poured into a gel block, a 20-prong comb was placed in to create wells and the bubbles removed with a pipette tip. After 30 minutes of cooling, the gel block was placed in a larger gel tank filled with TBE buffer, so the wells and surface of the gel were flooded. Eight µl aliquots of PCR products were mixed with 2 µl of loading dye on parafilm and injected into the wells once the comb was removed. Five µl of HyperLadder™ 1 kb was added to the far end lane. The electrophoresis gel was then run at 90 V for 1 hour or until the products had moved sufficiently far down the gel. A SynGene G-Box UV transilluminator was used to examine the gels under UV.

### 5.1.3 Sequencing methods

Two sequencing techniques were used to examine the DNA present within the brines, Illumina MiSeq for brine 215 and NextSeq for Billingham and 44 XC. This diversity of techniques is related to the time period over which the samples were collected and the techniques employed by the different funding bodies involved. MiSeq sequencing was carried out at Edinburgh Genomics, Edinburgh, UK and the NextSeq at Marine Biological Laboratory, Falmouth, USA. Blanks were also sent for amplicon sequencing, but were failed by the sequencing centre due to lack of DNA.

Like most modern sequencing techniques, Illumina MiSeq and NextSeq both begin with fragmenting the DNA into sizes of several hundred base pairs, repairing the ends and ligating

adapters to them. This DNA is then added to a flow cell which contains a primer field/anchors made up of sequences complimentary to the adapters. After hybridisation with this field, bridge amplification takes place which results in the strands bending over and hybridising with adjacent anchors. Repeating this results in clusters of sequences. Due to the bridge amplification, each cluster is composed of both forward and reverse orientated strands. Prior to sequencing, the reverse strands are removed. Sequencing occurs by hybridising the adapter sequence at the top of the attached DNA, to a sequencing primer. Then, 4 different fluorescently tagged nucleotides are added, bonding one at a time with their complimentary nucleotide in the DNA fragment. The excess nucleotides are washed away and the fluorescence of each cluster examined with a camera. The cycle is then repeated until each cluster has been sequenced. The chemistry involved between MiSeq and NextSeq is essentially the same, although the read length is long in MiSeq at 300 bp, compared to 150 bp in NextSeq, but the maximum output data is much higher in NextSeq. Both Illumina platforms can be used to sequence amplicons or entire environmental metagenomes (Voelkerding et al. 2009, Son et al. 2011). Table 5.3 contains the results of the MiSeq and NextSeq sequencing.

#### **5.1.4 Metagenomic data analysis**

Unassembled sequence files from the successfully sequenced metagenomes were compressed and uploaded to MetaGenome Rapid Annotation with Subsystem Technology (MG-RAST), an online metagenomics annotation server (Meyer et al. 2008). Data was uploaded in the FASTAQ format so that quality scores were retained and could be used for quality control. Prior to pipeline submission, end pair reads were joined using MG-RAST's integrated protocol.

A number of quality filtering options are available on the server. Sequences can be trimmed based on their phred score, replicate sequences removed and host specific sequences screened out. Initial results using default quality filters resulted in large sections of the metagenomes failing QC. To address this, the sequences were run through the pipeline six times each with different settings to determine what parts of the QC system were removing sequences. These runs involved tweaking the dynamic filtering setting (by phred score) and dereplication combinations. Dereplication works by comparing the first 50 characters of each sequence and if they are identical, assumes they are technical replicates and removes them. Dereplication is not appropriate for amplicon data, but is used when dealing with

metagenomic data sets. Dynamic filtering is based on Phred scores and (Ewing et al. 1998) determines the probability that each base was called correctly by algorithmically examining the shape of the peaks outputted by the sequencer. It then assigns a score based on a number of parameters such as the shape and resolution of the peak in question. The scale is logarithmic, with a score of 10 representing a base call accuracy of 90 % or a 1 in 10 chance of the base called being incorrect. This moves to 99 % or 1 in 100 at a Phred score of 20 (Ewing et al. 1998). Phred scores of 15 (MG-RAST default) and 3 were experimented with in this study. Screening for specific sequences was not implemented for any of the runs. This process allowed for an informed decision to be made on what quality control settings were best for further analysis, aiming to strike a balance between retaining reads post QC, and the quality of the reads remaining. Table 5.2 summarises these results.

The results at the end of the MG-RAST pipeline showed that the different QC settings had a variety of effects on the number of sequences removed. Dereplication is clearly the step most responsible for the large number of lost sequences in QC. 44 XC experienced the largest losses, with the number of sequences lost in QC (when using a phred score of 15) going from 11.4 % up to 80 %, a change of 68.6 %, when turning dereplication on and keeping all other settings identical. In comparison, the sequences lost in Billingham baths increased by 55.5 % with dereplication applied. 215 however, lost much fewer sequences, only moving from 4.2 % to 20 %. In contrast, moving the phred quality filtering scores to 3 from 15 only resulted in a loss of approximately 2-7 % of the sequences across the three datasets.

It was decided that dereplication was too important to the data quality to omit from the processing pipeline, particularly given Illumina's propensity to artificially replicate sequences. Additionally, the low diversity dataset meant that losing sequences was less important than in other high diversity datasets, since much of the community would be captured early into the sequencing.



	215						44 XC						Billingham Baths					
Number run through MG-RAST pipeline	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
Lowest phred score of 15					x	x					x	x					x	x
Lowest phred score of 3	x	x					x	x					x	x				
No dynamic trimming			x						x						x			
Dereplication turned on	x		x		x		x		x		x		x		x		x	
Dereplication turned off		x				x		x				x		x				x
Assembled				x						x						x		
Unassembled	x	x	x		x	x	x	x	x		x	x	x	x	x		x	x
% of sequences removed in QC	17.0	0.8	16.2	13.4	20.0	4.2	79.2	17.9	75.3	83.3	80.0	11.4	56.6	0.7	56.4	68.2	57.4	1.9

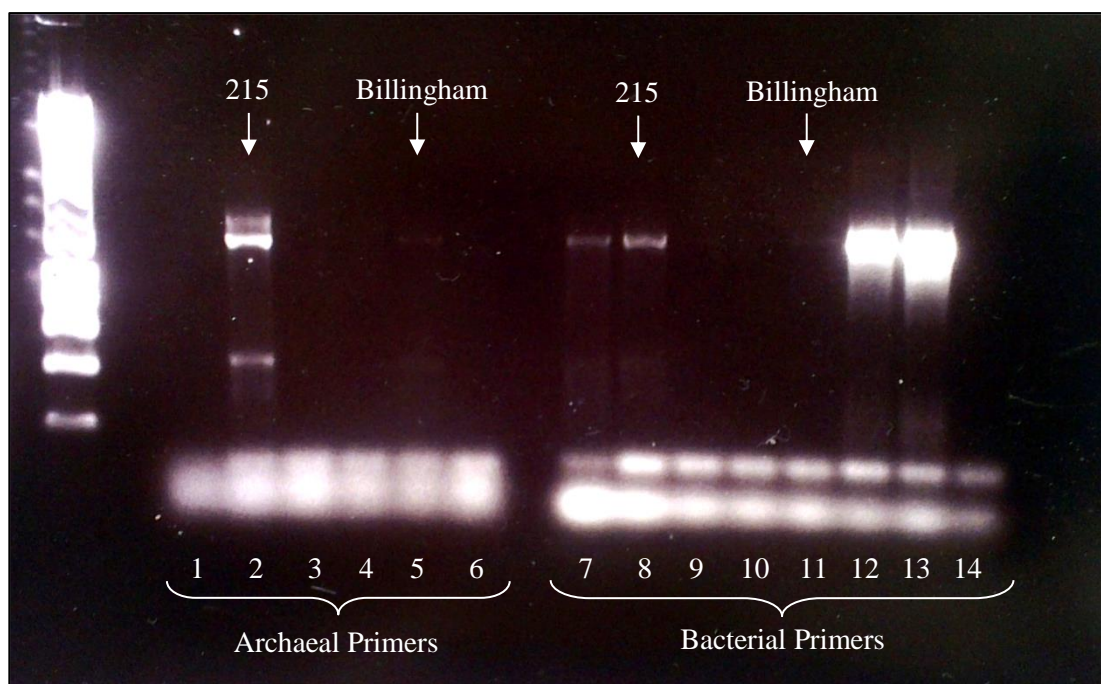
**Table 5.2.** The various quality control settings tested in MG-RAST for analysing the metagenomes from three brines, 215, 44 XC and Billingham Baths. The settings eventually used for analysis are highlighted in red. It appeared that the dereplication step was key to removing the majority of the sequences during quality control.

## 5.2 Results and discussion

### 5.2.1 DNA extraction

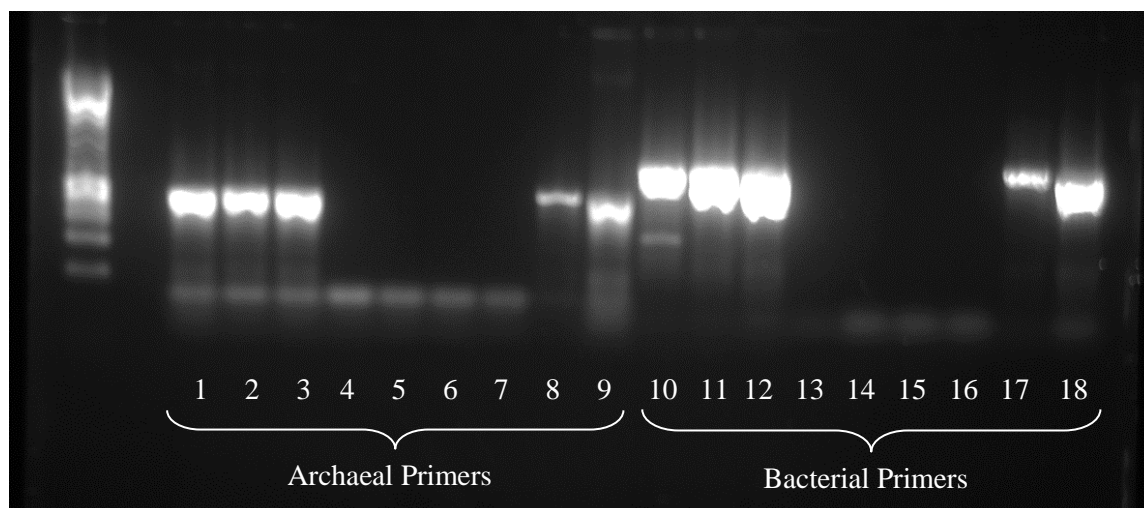
Extraction yields from all of the brines were exceptionally low. A NanoDrop Lite was initially used to check DNA quality and quantity in 1 µl aliquots. However, this proved ineffective. Readings were inconsistent, with blanks (molecular grade H<sub>2</sub>O) recoding higher levels of DNA than the samples, samples and blanks occasionally showing negative DNA concentrations and readings varying within identical samples. This was likely due to the extremely low DNA concentrations present in the samples, since the NanoDrop was tested with positive controls from the DNA extraction (brines spiked with *E. coli* DNA) and had performed well. Eventually the NanoDrop technique was abandoned for these samples. Instead, the outcome of the DNA extractions were only determined by PCR amplification and gel imaging.

Despite their reported usage in similar environments in the literature (e.g. Radax et al. 2001, Gramain et al. 2011, Park et al. 2009), the phenol chloroform extraction kits and additionally liquid nitrogen grinding consistently failed to extract DNA from the brines and rock salt samples. Both the MO BIO and fast prep kits with and without the liquid nitrogen grinding step also failed to produce any amplifiable DNA. However, when adding the phosphate buffer step to the MO BIO kit, amplifiable DNA was recovered from a number of brines (see Figure 5.1).



**Figure 5.1.** PCR products post gel electrophoresis imaged under UV from MO BIO PowerMax® Soil DNA Isolation Kit extraction with phosphate buffer step. Both archaeal (21F/958R) (samples 1-6) and bacterial (27F/1389R) (samples 7-14) primers were used for PCR. Amplification was successful in both brine 215 and Billingham Baths, but weaker in the latter. No amplification occurred in any of the solid salt samples. Positive and negative controls for both PCR and DNA extraction confirmed the results. Some non-specific binding occurred, especially at the base of each gel lane where two sets of primers dimers appear to be present. DNA extractions were also attempted on the solid salt samples in this image, as well as a positive control with bacterial specific primers. Key: 1. Soil control 2. Brine 215 3. Sylvinite 4. Halite 5. Billingham brine 6. Negative PCR control 7. Soil control 8. Brine 215 9. Sylvinite 10. Halite 11. Billingham brine 12. Positive control DNA extraction (*E. coli*) 13. Positive PCR control (*E. coli*) 14. Negative control (molecular H<sub>2</sub>O).

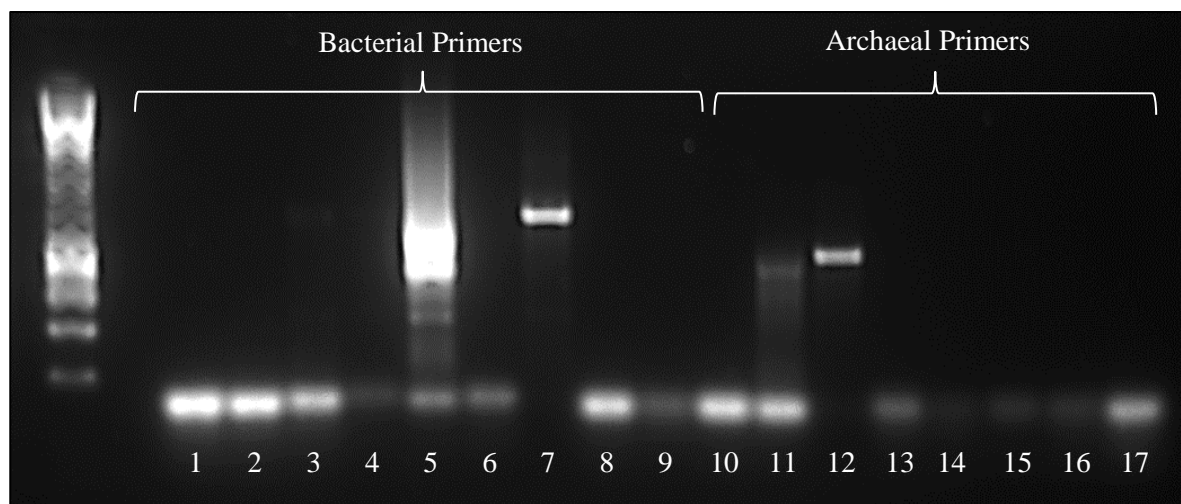
Despite success in 215 and Billingham, this protocol still was unable to produce amplifiable DNA from brines 44 XC, 29 XC and 101-P. DNA could be recovered when the brines were spiked with DNA before extraction with the MO BIO PowerMax® Soil DNA Isolation Kit with the additional phosphate buffer steps discussed in Section 5.1.2, suggesting the issue was not related to the brine geochemistry after phosphate buffer addition (see Figure 5.2).



**Figure 5.2.** PCR products post gel electrophoresis imaged under UV again with brine 44 XC, 101-P and 29 XC failing to produce products. However, the brines spiked with bacterial and archaeal DNA before extraction show PCR products. This shows that the DNA extraction methods used were not prevented from recovering DNA by the brine geochemistry or clay minerals. Both archaeal (21F/958R) (samples 1-9) and bacterial (27F/1389R) (samples 10-18) primers were used for PCR. Key: 1. Brine 29 XC spiked before extraction with *Haloferax volcanii* DNA 2. Brine 44 XC spiked before extraction with *Haloferax volcanii* DNA 3. Brine 101-P spiked before extraction with *Haloferax volcanii* DNA 4. Negative PCR control (molecular H<sub>2</sub>O) 5. Brine 29 XC 6. Brine 44 XC 7. Brine 101-P 8. Positive PCR control (*Haloferax volcanii*) DNA 9. Brine 44 XC spiked after extraction with *Haloferax volcanii* DNA 10. Brine 29 XC spiked before extraction with *E. coli* DNA 11. Brine 44 XC spiked before extraction with *E. coli* DNA 12. Brine 101-P spiked before extraction with *E. coli* DNA 13. Negative PCR control (molecular H<sub>2</sub>O) 14. Brine 29 XC 15. Brine 44 XC 16. Brine 101-P 17. Positive PCR control (*E. coli*) 18. Brine 44 XC spiked after extraction with *E. coli*.

Due to the success in extracting DNA from Billingham and 215 and the lack of issues in recovering spiked DNA, it was presumed that the problems with extraction from the other brines were related to a lack of biomass. Therefore, it was necessary to combine the DNA from multiple extractions and concentrate them. The recommended DNA concentration and clean up protocol in the MO BIO kit was found to be ineffective at concentrating DNA at low starting concentrations, which might be related to the low centrifugation speed (3000 g)

recommended. The adapted Urakawa et al. (2010) method outlined above (with linear acrylamide) was found to be sufficient to produce amplifiable DNA from one of the seeps (44 XC) after combining numerous extractions (see Figure 5.3).



**Figure 5.3.** PCR products post gel electrophoresis imaged under UV again with brine 44 XC producing a faint product and 101-P and 29 XC failing to produce products. Spiked samples show that the methods used to concentrate DNA did not result in the loss of amplifiable DNA or the introduce contamination. Archaeal (21F/958R) (samples 10-17) and bacterial (27F/1389R) (samples 1-9) primers were again used for the PCR. Key: 1. Negative PCR control (molecular H<sub>2</sub>O) 2. Brine 29 XC 3. Brine 44 XC 4. Brine 101-P 5. Positive PCR control (*E. coli*) 6. Blank collected in the field and run through all the DNA extraction and concentration steps 7. Positive DNA extraction and concentration control spiked with *E. coli* 8. Negative DNA extraction control (molecular H<sub>2</sub>O) 9. Negative DNA concentration extraction control (molecular H<sub>2</sub>O) 10. Negative PCR control (molecular H<sub>2</sub>O) 11. Brine 44 XC 12. Positive DNA extraction and concentration control spiked with *Haloferax volcanii* 13. Brine 29 XC 14. Brine 101-P 15. Negative DNA extraction control (molecular H<sub>2</sub>O) 16. Negative DNA concentration extraction control (molecular H<sub>2</sub>O) 17. Blank collected in the field and run through all the DNA extraction and concentration steps.

The combined DNA extractions from ~10 L of brine 44 XC concentrated down into 0.1 ml eventually resulted in enough DNA to generate a visible PCR product using the amplification cycles outlined in Section 5.1.2. Brines 29 XC and 101-P both continued to fail to produce amplification products, even when additional nested PCR cycles were done on

the same samples. The strongest products were generated with the archaeal primers in all the samples.

Overall, DNA was therefore successfully extracted from brines 215, Billingham baths and 44 XC. This consolidates the results of Chapter 4 in relation to 101-P, indicating that organisms were undetectable in the brine when using DNA as a proxy. The results also indicate that 29 XC, which was shown to be habitable in Chapter 4, does not contain extractable DNA or culturable organisms (using these methods). This suggests that the organisms present in brine were both not viable and/or so scarce that DNA extractions were unsuccessful. A potential explanation for the brine being geochemically being habitable, but containing extremely low concentrations of non-viable organisms could be related to the interplay between extremes found in the brine. This is discussed more in Chapter 4.

## **5.2.2 Metagenomics**

### **5.2.2.1 Metagenome characteristics**

Three metagenomes were successfully obtained from brines 215, Billingham and 44 XC. The 215 data set produced a total of 11.59 GB of sequences, Billingham produced 4.02 GB and 44 XC, 4.69 GB. These data were composed of 16,139,625 sequences in 215, 16,279,907 in 44 XC and 11,049,776 in Billingham Baths. Due to the different sequencing technologies used, sequence length was longer in 215 at  $344 \pm 75$  bp and shorter in 44 XC ( $122 \pm 49$  bp) and Billingham ( $161 \pm 26$ ). GC content was high in all the metagenomes at  $60 \pm 8$ ,  $57 \pm 15$  and  $64 \pm 7$  for 215, 44 XC and Billingham respectively.

As discussed in Section 5.1, quality control on the MG-RAST server resulted in the loss of significant numbers of sequences. It was eventually determined that this sequence loss was coming from the dereplication process. This process was considered too important to omit from processing given the sequencing technology and the low biomass samples. With these QC parameters, the actual data analysed was reduced significantly. 215 was reduced to 12,913,239 sequences, 44 XC to 3,251,444 and Billingham to 4,709,475. Sequence length and guanine-cytosine (GC) content were essentially unchanged. Predicted protein features post QC yielded 4,441,260 features in 215, 1,063,229 in 44 XC and 2,073,303 in Billingham. Predicted rRNA features in 215 numbered 392,211, 834,395 in 44 XC and 257,109 in Billingham. DRISSEE error values were fairly high at 37.351 % in 215, 9 % in Billingham and 4.868 % in 44 XC. However, since DRISSEE over estimates read errors in Illumina data,

these numbers were considered acceptable to proceed with (Eren *et al.* 2014). The general metagenome characteristics, pre and post QC are summarised on Table 5.3.

GC distribution in the three metagenomes was distinctly unimodal, with a single GC peak present in all metagenomes at around the 65-70% range. This is consistent with an environment dominated by extreme halophilic organisms which typically have GC content in the region of 57-68% (Paul *et al.* 2008).

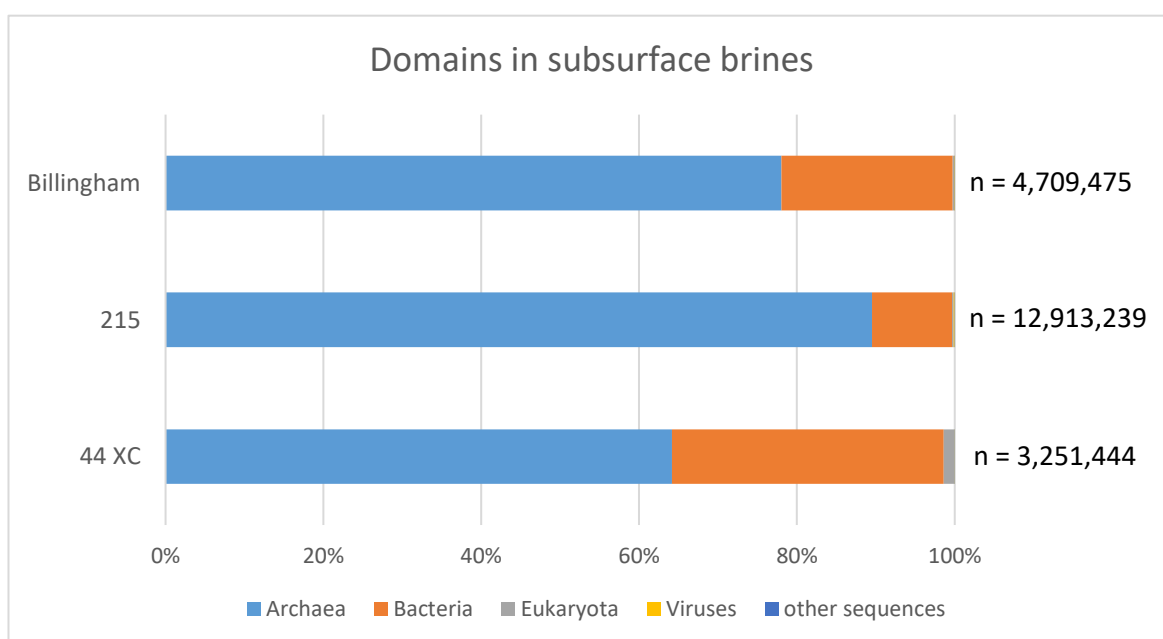
<b>Metagenome Name</b>	<b>215</b>	<b>44 XC</b>	<b>Billingham</b>
<b>MG-RAST ID</b>	4705070	4678909	4678908
<i>Pre QC</i>	-	-	-
<b>Base Pair Count (bp)</b>	5,559,605,502	1,989,994,878	1,789,443,230
<b>Sequence Count</b>	16,139,625	16,279,907	11,049,776
<b>Mean Sequence Length (bp)</b>	344 ± 75	122 ± 49	162 ± 27
<b>Mean GC (%)</b>	61 ± 8	57 ± 16	64 ± 7
<i>Post QC</i>	-	-	-
<b>Base Pair Count</b>	3,984,059,653	351,915,600	1,789,443,230
<b>Sequence Count</b>	12,913,239	3,251,444	4,709,475
<b>Mean Sequence Length (bp)</b>	309 ± 109	108 ± 46	161 ± 37
<b>Mean GC (%)</b>	61 ± 8	60 ± 10	64 ± 7
<b>Predicted Protein Features</b>	4,441,260	1,063,229	2,073,303
<b>Predicted rRNA Features</b>	392,211	834,395	257,109
<b>Identified Protein Features</b>	2,160,413	337,729	899,048
<b>Identified rRNA Features</b>	2,548	1,538	1,427
<b>Predicted Subsystems Functions</b>	7,188,175	670,157	2,310,854

**Table 5.3.** General metagenome characteristics for all three brines, with both pre- and post-quality control (QC) data. Brine 215 was sequenced using MiSeq and 44 XC and Billingham using NextSeq. For this reason, the number of sequences generated was higher for 215.



### 5.2.2.2 Taxonomic diversity

Metagenomic sequencing of the DNA extracted from brines 44 XC, 215 and Billingham Baths reveals three similar microbial communities. At the domain level, all three brines are dominated by archaeal sequences, with some variability between the brines. In the Billingham brine, 78 % of the sequences were identified as archaeal, compared to 64 % in 44 XC and 90 % in 215. Bacterial sequences again show some variability, making up 22 %, 34 % and 10 % of the total sequences in Billingham, 44 XC and 215 respectively. Eukaryotic sequences are only minor components of each brine, 0.2 % in both Billingham and 215 and higher in 44 XC at 1 %. The proportions of these domains are fairly typical for extreme NaCl brines (see Section 5.2.3.3). Numbers of sequences not assigned to any of the major divisions within RefSeq were relatively consistent between the metagenomes, with 0.1 % in 44 XC, 0.07 % in Billingham and 0.06 % in 215.

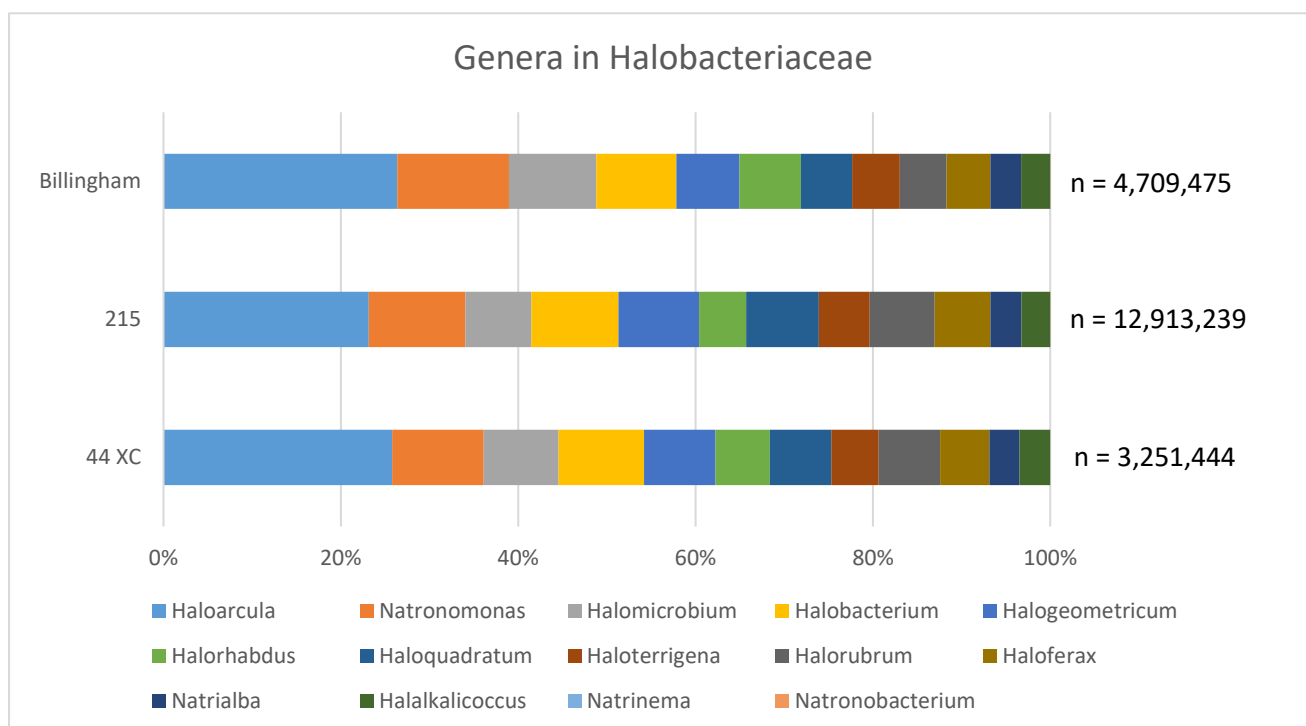


**Figure 5.4.** The overall taxonomic structure of the three brines by domain as a percentage abundance. Archaea dominate all three of the brines. Bacteria make up 10 % to 34 % of the brines. Eukaryotes and virus are the minority of sequences in all three. Note the different total sequence numbers for each.

The archaeal community in all three deep subsurface brines is essentially exclusively composed of Euryarchaeota. Crenarchaeota, the second most abundant phylum, only make up 0.4 %, 0.1 % and 0.2 % of 44 XC, Billingham and 215. The Euryarchaeota phylum is also

low diversity, being almost completely dominated by one family of organisms, the *Halobacteriaceae*.

As discussed in Chapter 2, the Halobacteriaceae family are specifically adapted to extreme salinity and typically dominate mature extreme hypersaline environments. Within this family, a range of different genera are present. *Haloarcula* was the most dominant genera in all three brines, making up 23 % of the hits within Halobacteriaceae in 215 and 19 % of the overall sequences. Billingham was similar, with *Haloarcula* making up 26 % of the hits within Halobacteriaceae and 20 % of the overall sequences. 44 XC has a similar proportion of *Haloarcula* 26 % within *Halobacteriaceae*, but a lower overall proportion of 16 % since the archaeal population makes up less of the microbial community than in the other two brines. The other top five genera included *Natronomonas* (44 XC, 10 %, Billingham, 13 % and 215, 11 % in *Halobacteriaceae*), *Halogeometricum* (44 XC, 8 %, Billingham, 7 % and 215, 9 %), *Halomicrobium* (44 XC, 8 %, Billingham, 10 % and 215, 7 %) and *Halobacterium* (44 XC, 10 %, Billingham, 9 % and 215, 10 %). The proportions of these varied slightly between the brines, Billingham for example had slightly higher portions of *Halomicrobium* and *Natronomonas*, and lower proportions of *Halogeometricum*, but generally the genus distribution between the brines was remarkably consistent within this group of organisms. Other studies have compared Mediterranean and Atlantic saltern concentrator ponds, finding changes between dominant genera between salterns, such as a change from *Halorubrum* to *Haloquadratum* (Fernández et al. 2014). Here, the dominant genera are consistent.



**Figure 5.5.** Genera present in the Halobacteriaceae family as percentage abundance in the three brines. *Haloarcula* appears to be the dominant genus in Halobacteriaceae in all three brines at 23-26 %, followed by *Natronomonas* at 10-13 %. Note the different total sequence numbers for each metagenome.

Another group of organisms of interest present within Euryarchaeota is the Methanomicrobia order, which make up a small number of total (~0.3-0.9 %) of the reads within Euryarchaeota. In the three brines, this order includes organisms from genera such as *Methanosarcina*, *Methanococcoides* and *Methanoculleus*. Methanomicrobia are of interest due to their ability to produce methane that can be used as a carbon and energy source by certain organisms. However, based on the work carried out on the gases trapped in the salt rocks from Chapter 3, it appears they are not responsible for the trapped methane found in the sylvinite since that is clearly of thermogenic origin.

A small number of archaeal hits fell into other phyla, the most in Crenarchaeota, followed by very small numbers of hits in Thaumarchaeota (215, 0.02 %, 44 XC, 0.06 %, Billingham, 0.1 % of archaeal hits), Korarchaeota (215, 0.009 %, 44 XC, 0.04 %, Billingham, 0.007 % of archaeal hits) and Nanoarchaeota (215, 0.008 %, 44 XC, 0.03 %, Billingham, 0.002 % of archaeal hits). Within Crenarchaeota, Thermoprotei were the only class identified, within which hits were attributed to several different orders, such as Desulfurococcales, Thermoproteales and Sulfolobales (in abundance order in all three metagenomes).

The similarity of the archaeal communities in the three brines is surprising, particularly between 44 XC and the other two brines. As shown in Chapter 3, all three brines originated in the Sherwood Sandstone aquifer. However, the brines 215 and Billingham appear to have taken a different pathway through the deep subsurface by exploiting a fault down from the Sherwood Sandstone. In contrast, 44 XC spent more time in the evaporite deposit and has clearly interacted with a greater range of salt minerals giving it a distinctly higher Mg content. Additionally, Billingham had likely been exposed to oxygen in the mine air for a reasonable period. 44 XC was sampled directly from a wall seep, with oxygen exposure only occurring during sampling. This would not have had time to alter the community present since the samples were immediately chilled and then frozen shortly after. These differences in environmental conditions appear to have had very little impact on the archaeal community structure.

The major difference between the three brines is their variation in bacterial/archaeal content (bacteria make up 22 %, 34 % and 10 % in Billingham, 44 XC and 215). It is not clear why this difference exists since they are geochemically similar brines. 44 XC has a higher Mg content and was sampled directly from a tap and so had minimal contact with oxygen. However, 215 also had very little time between oxygen exposure and sampling, and displayed much lower bacterial content. This aspect may be related to the different migration pathway taken by 44 XC through the deep subsurface, meaning it encountered and entrained different organisms from outside the evaporite deposit.

The top bacteria phyla included Proteobacteria (215, 37 %, 44 XC, 66 %, Billingham, 61 % of bacterial hits), Bacteroidetes (215, 13 %, 44 XC, 5 %, Billingham, 15 % of bacterial hits), Firmicutes (215, 17 %, 44 XC, 11 %, Billingham, 7 % of bacterial hits), Actinobacteria (215, 14 %, 44 XC, 11 %, Billingham, 7 % of bacterial hits) and Chloroflexi (215, 6 %, 44 XC, 1 %, Billingham, 2 % of bacterial hits).

Several halophilic organisms exist in the bacterial hits. Most prominent is the *Salinibacter* genus, which almost totally dominates the phylum Bacteroidetes (215, 1 %, 44 XC, 1 %, Billingham, 2 % of total hits). This genus contains extremely halophilic bacteria uniquely adapted to saline environments (Antón et al. 2002, Makhdoumi-Kakhki et al. 2012). In some mature saturated NaCl brines in salterns, *Salinibacter* species have been shown to compose up to 5 and 25 % of the total prokaryotic community (Antón et al. 2002). A number of other bacteria genera, including *Oceanobacillus* (215, 0.02 %, 44 XC, 0.09 %, Billingham, 0.02 % of total hits) and *Geobacillus* (215, 0.1 %, 44 XC, 0.1 %, Billingham, 0.1 % of total hits) also have been shown to contain a number of moderate halophiles.

A handful of genera composed of thermophiles are found in the brines. These include *Geobacillus* (215, 0.1 %, 44 XC, 0.1 %, Billingham, 0.1 % of total hits) and *Rubrobacter* (215, 0.2 %, 44 XC, 0.2 %, Billingham, 0.2 % of total hits). The native rock temperature in the mine is too low for thermophiles, making their presence in the metagenome puzzling. However, previous studies have found thermophiles such as *Geobacillus* in a variety of mild environments (Zeigler 2014). It is possible that these thermophiles have been transported up into the evaporite deposit from deeper, hotter regions of the deep subsurface. Alternatively, closely related organisms may not have the same thermal tolerance, so these organisms could actually be adapted to the lower temperatures in the brines.

*Geobacter* was detected in all three of the brines (0.1 % in Billingham, 0.1 % in 44 XC and 0.1 % in 215 of total metagenome hits). This genus consists of anaerobic chemoorganotrophic mesophiles with the distinctive ability to reduce insoluble Fe(III) and Mn(IV) (Röling 2014). Although there is less than 50 mg/l of Fe present in the brines (see Chapter 3), iron reduction could still take place at low rates. However, *Geobacter* do not appear to be adapted to grow at the salinities observed in the brines (e.g. Nevin et al. 2005, Röling 2014, Sun et al. 2014). These organisms may have been active when the fluid was more dilute, but have been entrained and transported into the evaporite sequence. This means they are unlikely to be active whilst in the brines. Other iron reducers exist in the *Desulfuromonas* genus (0.02 % in Billingham, 0.02 % in 44 XC and 0.01 % in 215 of total metagenome hits), which includes species shown to reduce iron and can have higher salinity tolerances (e.g. Badalamenti et al. 2016).

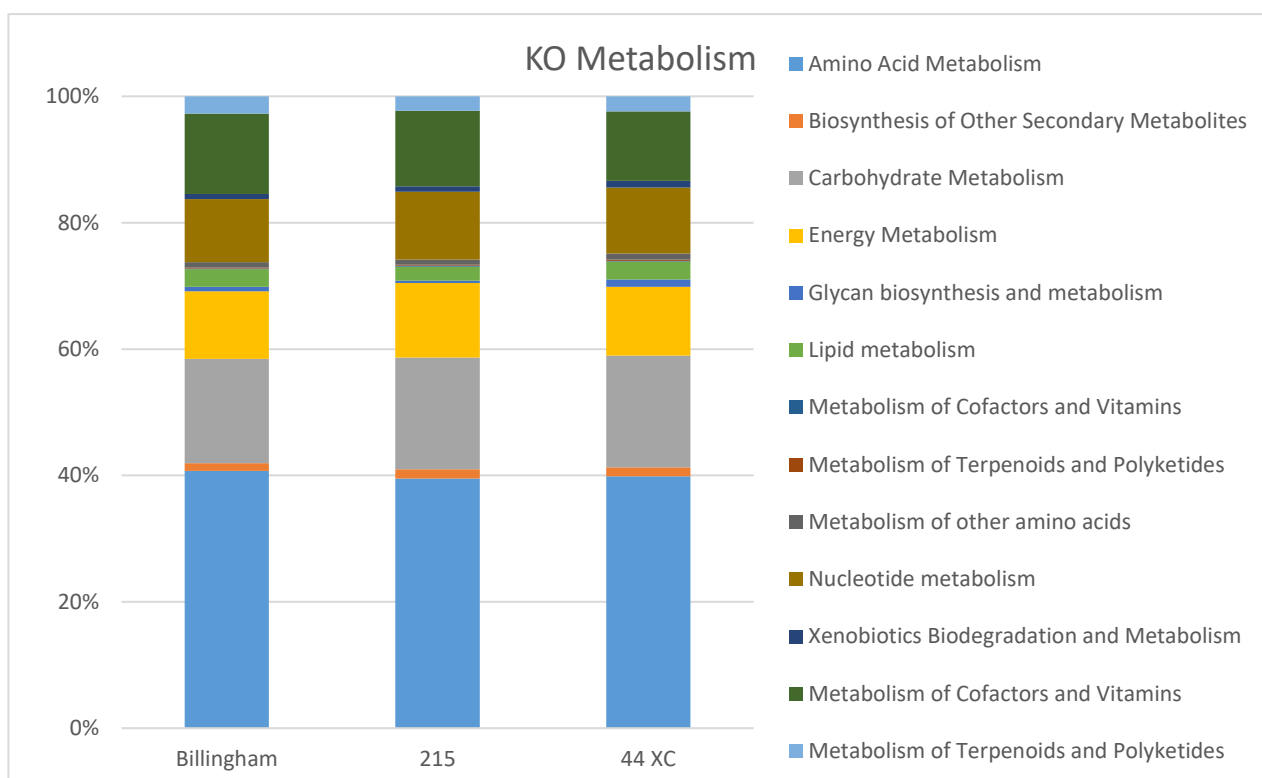
Hits in the *Desulfovibrio* genus were found in all three brines (0.2 % in Billingham, 0.1 % in 44 XC and 0.08 % in 215 of total metagenome hits). This genus is composed of organisms able to use sulphate as an electron acceptor (Voordouw 1995). Several species in the genus are halophilic (e.g. Caumette et al. 1991, Tardy-Jacquenod et al. 1996, Thabet et al. 1996). Hits were also found for the *Desulfohalobium* genus in all three brines (0.08 % in Billingham, 0.03 % in 44 XC and 0.02 % in 215 of total metagenome hits), which also display similar sulphate reduction abilities and halophilic modes of life (e.g. Ollivier et al. 1991, Jakobsen et al. 2006). Sulphate is present in the brines in moderate concentrations (~2-4 g/l, see Chapter 4) and sulphate minerals are found in regions above and below the mine. The metabolic pathways related to these types of organisms are discussed below.

Fungi are a common feature of extreme saline environments. The metagenomes display a number of different genera which have been identified in other hypersaline environments around the world, although the numbers of hits for these organisms in all three metagenomes

was low. The most dominant genus was *Aspergillus* (215, 0.003 %, 44 XC, 0.009 %, Billingham, 0.004 % of total hits), with a few other hits being attributed to genus such as *Emericella* and *Penicillium*. These groups of organisms have been identified in other surface hypersaline environments (Gunde-Cimerman and Zalar 2014).

### **5.2.2.3 Functional diversity**

SEED Subsystems and KEGG mapping were used to examine the functional gene profile on the MG-RAST server. This generated 4,279,250, 1,402,652, 424,227 hits in the Subsystems database from 215, Billingham and 44 XC respectively. Housekeeping functional groups including protein (215, 10.06 %, 44 XC, 10.83 %, Billingham, 10.36 % of protein hits) amino acids (215, 9.99 %, 44 XC, 10.51 %, Billingham, 11.12 % of protein hits) and carbohydrate (215, 9.22 %, 44 XC, 9.87 %, Billingham 9.55 % of protein hits) metabolism were the most abundant. Focus here will be given to genes involved in metabolic processes to help determine how organisms are acquiring carbon and energy in these deep subsurface environments. The pathways discussed below were identified using both Subsystems and KEGG and the numbers reported are from KEGG, unless otherwise specified.



**Figure 5.6.** Proportions of general KO (KEGG Orthology) metabolic categories identified within the metagenomes sequenced from the three Boulby brines.

#### 5.2.2.3.1 Nitrogen Metabolism

The majority of halophiles prefer to utilise aerobic respiration for energy acquisition due to it being the most energetically productive metabolism. The abundance of cytochrome c oxidase (EC 1.9.3.1) related genes in the three metagenomes supports this. However, in the deep subsurface, oxygen supplies are often limited. Other metabolisms would need to replace aerobic respiration to become the primary means of generating ATP when oxygen levels are low.

Denitrification is a metabolism several halophiles have been demonstrated to be capable of and can function at NaCl saturation (Oren 2011b, 2013). The genes coding for enzymes involved in denitrification, including nitrate reductase (EC 1.7.99.4; 215, 3221 hits, Billingham, 1653 hits, 44 XC, 294 hits), nitrite reductase (EC 1.7.2.1; 215, 7354 hits, Billingham, 2147 hits, 44 XC, 358 hits), nitric oxide reductase (EC 1.7.2.5; 215, 9395 hits, Billingham, 2405 hits, 44 XC, 540 hits) and nitrous-oxide reductase (EC 1.7.2.4; 215, 1845 hits, Billingham, 580 hits, 44 XC, 243 hits) are all present in the three metagenomes. The top

taxa involved include organisms from genera such as *Haloarcula*, *Halomicrobium*, *Halorhabdus* and *Halogeometricum*. The high number of hits associated with these stages in all three metagenomes, suggests this is an important metabolism in the brines. In low oxygen environments, denitrification provides a metabolism capable of producing close to the amount of free energy of aerobic respiration. It therefore would be a viable alternative in oxygen starved hypersaline environments, where highly energetic metabolisms are favoured. Nitrate was found in 44 XC at 560 mg/l, indicating the ion is present in certain brines in the evaporite deposit, despite being below detection limits in the other brines and generally proving scarce in hypersaline environments (Oren 2013).

Genes involved in assimilatory nitrate reduction such as ferredoxin-nitrite reductase (EC 1.7.7.1) were also abundant in all three metagenomes. Small numbers of genes coding for enzymes involved in dissimilatory nitrate reduction such as nitrite reductase (NAD(P)H) (EC 1.7.1.4) were present in the metagenomes and were attributed mainly to bacterial genera such as *Nocardiopsis* and *Marinobacter*, although these species were not consistent across the three metagenomes.

#### 5.2.2.3.2 Sulphur Metabolism

Enzymes responsible for sulphate reduction were detected in all three metagenomes to varying proportions. These included sulfate adenylyltransferase (EC 2.7.7.4; 215, 1180 hits, 44 XC, 231 hits, Billingham, 376 hits), adenylyl-sulfate kinase (EC 2.7.1.25; 215, 256 hits, 44 XC, 54 hits, Billingham, 90 hits) and phosphoadenylyl-sulfate reductase (EC 1.8.4.8; 215, 7693 hits, 44 XC, 601 hits, Billingham, 1947 hits).

Genes coding for adenylyl-sulfate reductase (EC 1.8.99.2; 215, 4 hits, 44 XC, 0 hits, Billingham, 37 hits) and dissimilatory sulfite reductase (EC 1.8.99.5; only 7 hits in Billingham), all of which are key to dissimilatory sulphate reduction, are uncommon or absent from the metagenomes. Billingham appears to be the only metagenome with the complete pathway present. Where they are found, dissimilatory sulfite reductase and adenylyl-sulfate reductase hits are attributed to genera, *Desulfohalobium*, *Desulfomicrobium*, *Desulfovibrio* and *Desulfomicrobium*.

This detection of partially complete sulphur cycles has been found before in hypersaline environments (e.g. Fernández et al. 2015). Whilst it may be related to a lack of sampling depth, saturation levels of NaCl appears to limit dissimilatory sulphate reduction (Oren 2011b). The small numbers of sulphate reducers identified in the taxonomy may have simply been entrained in the fluids during their passage through other parts of the deep subsurface.



Overall, sulphate reduction as an energy acquisition tool appears to be of minor importance in these brines and their surface counter parts (see Section 5.2.3.2 for more).

#### 5.2.2.3.3 Fermentation

Fermentation also provides an alternative metabolism when environmental oxygen concentrations are low and appears to be an important metabolism in the three metagenomes. Genes coding for key enzymes involved in fermentative arginine degradation (ADI pathway) are found in the three metagenomes, such as arginine deiminase (EC 3.5.3.6; 215, 21 hits, Billingham, 184 hits, 44 XC, 29 hits), carbamate kinase (EC 2.7.2.2; 215, 857 hits, Billingham, 253 hits, 44 XC, 100 hits), and catabolic ornithine transcarbamylase (EC 2.1.3.3; 215, 2983 hits, Billingham, 1096 hits, 44 XC, 388 hits). These were attributed to organisms like *Salinibacter*, *Pseudomonas* and *Halobacterium*. This pathway has been identified previously as a method used by *Halobacterium salinarum* to deal with oxygen limited conditions (Baliga et al. 2002, Wimmer et al. 2008). Enzymes involved in arginine production by fermentation and homolactic and ethanol fermentation were also present in the three metagenomes.

#### 5.2.2.3.4 Carbon acquisition

Many halophiles are chemoheterotrophic (Oren 2013) and most members of the Halobacteriaceae genus can metabolise a range of simple carbon compounds (Oren 2006). It is therefore unsurprising to find a range of different basic carbohydrate usage pathways present. Various pathways related to the metabolism of many monosaccharide and polysaccharides were detected in all three metagenomes, including complete pathways for galactose, fructose, mannose and xylose use, related to genera such as *Halorhabdus*, *Halomicrobium*, *Chromohalobacter*, *Halomonas*. Genes related to the utilisation of amino acids including, alanine, arginine, glycine, isoleucine and valine related to *Natromonous* and *Haloarcula*, were also present amongst others.

Reduction of fumarate could be an important metabolism in helping halobacteriaceae survive in low oxygen conditions (Oren 2013). Fumarate reductase (frdABCD EC 1.3.5.4; 215, 480 hits, Billingham, 46 hits, 44 XC, 70 hits), a key enzyme in this process is found in the metagenomes, associated predominantly with *Halorubrum*. Trimethylamine N-oxide (TMAO) reductase (EC 1.7.2.3), another potential anaerobic metabolism (Oren 2013), was absent in the three metagenomes aside from a small number of hits (9) in 215 related to the *Escherichia*.

In the deep subsurface, recalcitrant carbon could be important for microbial communities when acquiring carbon for growth (e.g. Purkamo et al. 2015). Of potential importance to this deep subsurface environment is the presence of hydrocarbons (see Chapter 6), as a clear communication exists between oils from the underlying carboniferous deposit and overlying Permian evaporites at Boulby, as demonstrated by the hydrocarbons sampled at Boulby (see Section 6.1.1). Unfortunately, little is known regarding the molecular pathways of hydrocarbon degradation in halophiles. From the small number of studies available, it appears pathways in which these metabolisms occur could be similar to those found in non-halophiles (Fathepure 2015).

Overall, little evidence of significant hydrocarbon usage is present in the three brine metagenomes. Often pathways were either incomplete using either KEGG or Subsystems, or contained very low very small numbers of hits (often just 1 or 2) spread inconsistently over a handful of bacterial genera. Genes coding for enzymes like catechol 1,2-dioxygenase (EC 1.13.11.1) or protocatechuate 3,4-dioxygenase (EC 1.13.11.3), which participate in the degradation of aromatic compounds and have been found previously in phenol degrading halophilic bacteria like *Halomonas organivorans* (Bonfá et al. 2013), were observed in small numbers of bacterial hits from organisms such as such as *Pseudomonas*. Alkane hydroxylases, important enzymes in the degradation of alkanes (Nie et al. 2014), were detected in small numbers of hits. Alkane 1-monooxygenase (EC 1.14.15.3) was observed in KEGG, again related to bacteria such as *Pseudomonas*, except for in Billingham, where most of the hits (28) were related to *Alcanivorax*, an important oil degrading organism (Golyshin et al. 2003). Key genes involved in the degradation of polycyclic aromatic hydrocarbons (PAHs), such as naphthalene dioxygenase (EC 1.14.12.12) were absent from the three metagenomes.

Another recalcitrant carbon source potentially available in the evaporite deposit is cellulose. Cellulose fibres have been shown to exist in rock salt (Griffith et al. 2008). Endoglucanase and beta-glucosidase both involved in the degradation of cellulose were scarce in the Subsystems analysis, aside from a small number of hits attributed to *Geobacter* and *Pelobacter* in the Billingham metagenome, but were present in all three metagenomes in the KEGG analysis (Endoglucanase, EC 3.2.1.4, 215, 1551 hits, Billingham, 4647 hits, 44 XC, 302 hits and beta-glucosidase, EC 3.2.1.21; 215, 26 hits, Billingham, 7 hits, 44 XC, 27 hits). Top hits came from *Halogeometricum*, *Natronomonas* and *Haloarcula*. Halophiles from deep subsurface salt deposits have been shown previously to be able to degrade cellulose

(e.g. Vreeland et al. 1998), and so the presence of pathways related to cellulose degradation is consistent with previous studies.

Methanotrophs appear largely absent from the three brines. Key genes for the serine pathway, ribulose monophosphate and xylulose monophosphate pathways were not found in the three metagenomes in using both KEGG and Subsystems. This may indicate that despite the presence of thermogenic methane in the salt (Chapter 3), the communities present in the brines do not appear able to use it as a major energy source.

Despite the identification of small populations of methanogens in the taxonomic analysis, a key enzyme involved in the three types of methanogenesis, methyl-coenzyme M reductase (EC 2.8.4.1) (e.g. Friedrich 2005), was absent from all three metagenomes whilst others like heterodisulfide reductase (EC 1.8.98.1) were present in all three, related to organisms such as *Methanopyrus*. It is hard to understand why methyl-coenzyme M reductase (EC 2.8.4.1) was consistently absent in both KEGG and SEED annotations across all three metagenomes, particularly since heterodisulfide reductase (EC 1.8.98.1) acts in partnership with methyl-coenzyme M reductase (EC 2.8.4.1) (Hedderich et al. 2005).

Although many halophiles are heterotrophic, autotrophic organisms do exist in surface hypersaline environments, the majority of which are phototrophs. These include various groups such as cyanobacteria, algae and anoxygenic phototrophic bacteria, as well as sulphur-oxidizing bacteria (SOB) such as *Thiohalorhabdus denitrificans* (Sorokin et al. 2008, Maheshwari and Saraf 2015).

Some genes related to carbon fixation are present in the metagenomes. RuBisCo is a critically important enzyme involved in photo- and chemoautotrophic CO<sub>2</sub> fixation in a range of different proteobacteria (Badger and Bek 2008), and has been found in other deep subsurface environments (e.g. Alfreider et al. 2009). RuBisCo was present (215, 3424 hits, Billingham, 906 hits, 44 XC, 228 hits) related to organisms such as *Natronomonas* and *Halomicrobium*. RuBisCo comes in several forms, type III being found primarily in archaea. This form of RuBisCo is involved in light-independent reactions that enable CO<sub>2</sub> to be incorporated into sugars (Sato et al. 2007, Tabita et al. 2007, Wrighton et al. 2016), but still requires light to function. Whilst RuBisCo's ability to function without light could be misunderstood, the complete lack of light in the deep subsurface means it is unlikely that it is being used to fix CO<sub>2</sub>, despite its presence in the metagenomes.

A number of key genes involved in the reverse TCA cycle were also present in all three metagenomes. These being citrate synthase (EC 2.3.3.1, 215, 9085 hits, Billingham, 2491

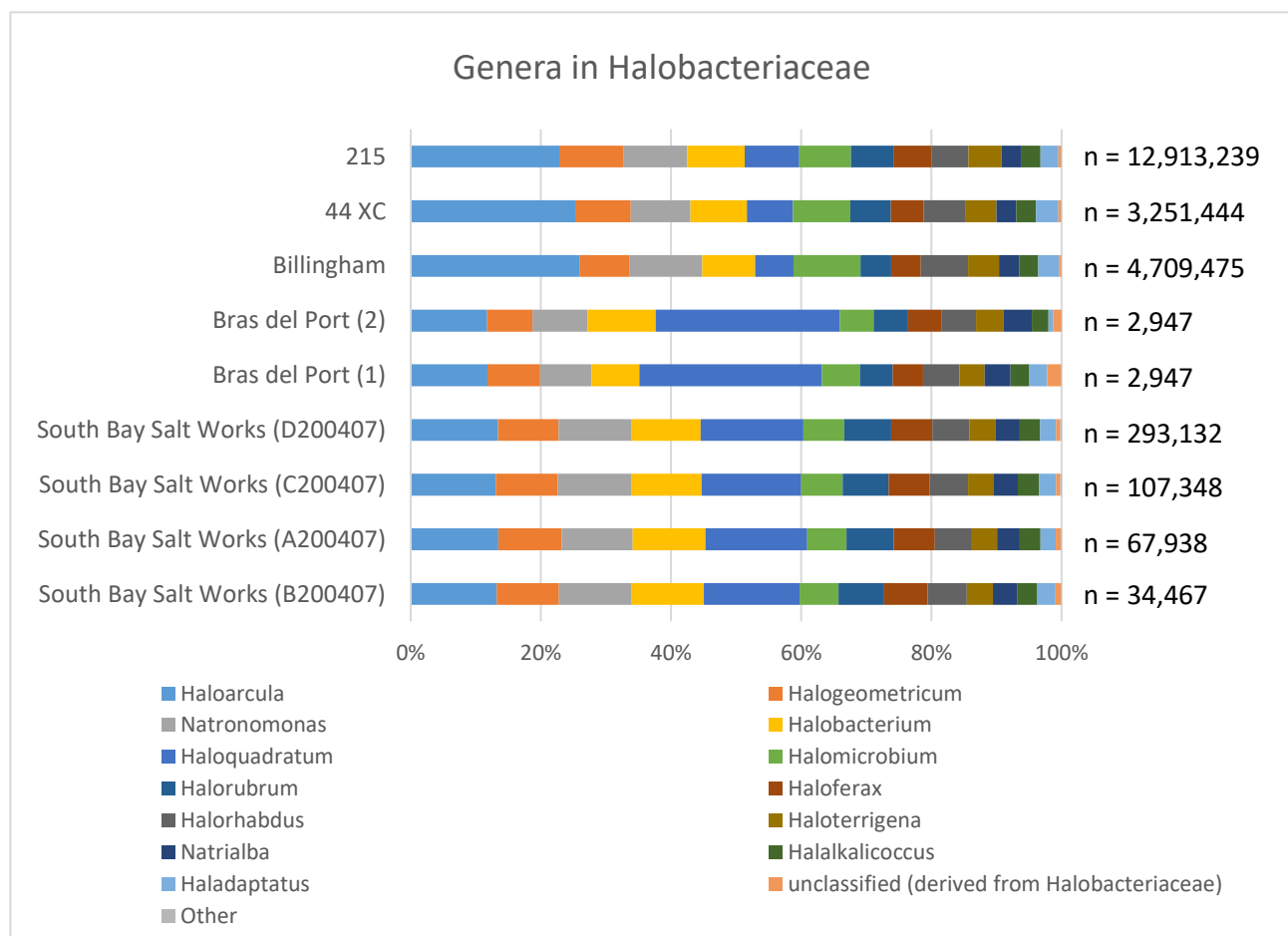
hits, 44 XC, 1029 hits), 2-oxoglutarate:ferredoxin oxidoreductase (EC 1.2.7.3, 215, 18768 hits, Billingham, 5127 hits, 44 XC, 1217 hits), pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1 215, 149 hits, Billingham, 125 hits, 44 XC, 10 hits), although a key enzyme ATP citrate lyase (EC 2.3.3.8) was absent. The key enzyme involved in the Wood-Ljungdahl pathway, carbon monoxide dehydrogenase/acetyl-CoA synthase subunit alpha (EC:1.2.7.4, 1.2.99.2, 2.3.1.169) (Håvelsrud et al. 2013), was present, but only with small numbers of hits in Billingham (31 hits) and 215 (7 hits). It is worth noting the absence of this enzyme in 44 XC could be due to the lower number of sequences generated for this sample. The hits were related to *Desulfatibacillum*, *Syntrophobacter* and *Desulfococcus*, amongst others. Other important parts of the Wood-Ljungdahl pathway, such as NADP-dependent formate dehydrogenase (EC 1.2.1.43), were, however, absent from the metagenomes. Key enzymes such as, 3-hydroxypropionate dehydrogenase, involved in the 3-hydroxypropionate cycle, were not detected. With key enzymes missing and RuBisCo probably unable to function in the deep subsurface, it appears autotrophy is not an important part of the community in these brines.

#### 5.2.2.4 Comparison to surface environments

To compare the differences between the deep subsurface brines from this study and surface examples, six other publicly available metagenomes were examined. These were taken from mature saturated NaCl extreme brines, two from Bras del Port, Santa Pola, Spain (Rodriguez-Valera, MG-RAST ID's 4441050.3, 4442451.3) and four from South Bay Salt Works, Chula Vista, CA, USA (Rodriguez-Brito, MG-RAST ID's 4440438.3, 4440433.3, 4440429.3, 4440430.3).

All are again dominated by archaeal sequences, making up 70-82 % of the community and bacterial sequences ranging from around 10-20 %. These are very similar proportions to those found in Billingham and 215. Brine 44 XC is comparable, but has a slightly higher bacterial content. Halobacteriaceae also dominate the surface brines. In both sets of surface brines, *Haloquadratum* is the domain genera, although it is worth noting that the metagenomes from Bras del Port were taken from samples deliberately sampled to include high numbers of *Haloquadratum*. *Haloarcula*, the dominant genera in the subsurface brines, is the second most common genera in the surface brines, followed by *Natronomonas*, *Halogeometricum* and *Halobacterium*. Despite some differences in relative abundance in a couple of genera, the proportions of the top genera between the surface and subsurface brines are similar within this family (see Figure 5.7). It is difficult to determine exactly what

controls these similarities and differences in dominant genera, something that other authors have also struggled with (e.g. Fernández et al. 2014), but one possible explanation is discussed further down in this section. It is interesting that genera such as *Halorhabdus*, which contains the species *Halorhabdus tiamatea*, an organism known to grow poorly under oxic conditions but thrives in hypoxic conditions (Antunes et al. 2008b), was not over represented in the subsurface brines relative to those at the surface.



**Figure 5.7.** Genera present in the *Halobacteriaceae* family between as a percentage abundance in Boulby, Bras del Port and South Bay Salt Works metagenomes. *Haloarcula* is dominant genus in all three subsurface brines at 23-26 % and *Haloquadratum* at 28-15 % in the surface metagenomes. Note the different total sequence numbers for each.

Methanomicrobia were also found in the surface brines. The population was larger in all six surface brines, ranging from 1.6-2.3 % compared to the ~ 0.3-0.8 % in the deep subsurface brines, suggesting methanogenesis is less prevalent in the Boulby brines in comparison to the surface brines.

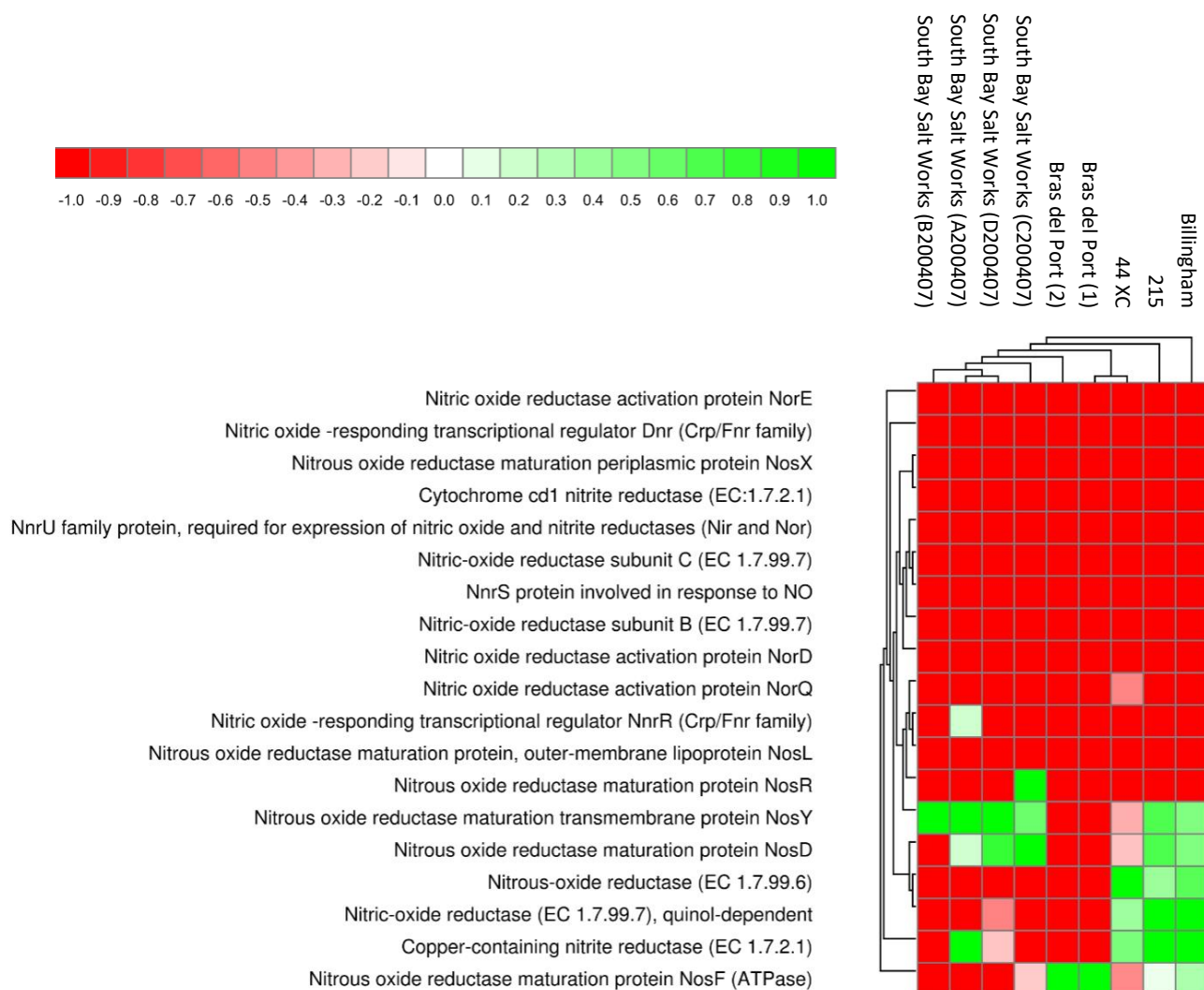
At a higher level, the bacterial population between the surface and deep subsurface brines are comparable. The top five phyla are close to identical, and include Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria making up four of the subsurface and surface top five. The fifth member of that top five in the subsurface brines is Chloroflexi and Cyanobacteria in the surface brines. Cyanobacteria were found in the subsurface metagenomes, but in proportionally smaller amounts, as has been observed in other deep subsurface metagenomes (e.g. Kormas et al. 2003, Miettinen et al. 2015). The role of such organisms in the deep subsurface is currently unclear (Miettinen et al. 2015). Bacteroidetes appear to be far more common in the South Bay Salt Works metagenomes, making up over ~ 61-57 % of the bacterial sequences, in comparison to the ~ 26-25 % in the Bras del Port metagenomes and ~ 18-6 % in the deep subsurface brines. Proteobacteria levels are comparable between 215 and the Bras del Port metagenomes (~ 29-35 %), but are higher in 44 XC and Billingham (~ 66-60 %).

The *Salinibacter* genus was again prominent in the surface brines. The genus was more common in the South Bay Salt Works metagenomes at ~ 8-10 % of the total hits compared to ~ 1-3 % in the deep subsurface brines. In contrast the Bras del Port metagenomes had comparable proportions of *Salinibacter* to the deep subsurface brines at ~ 2 % of the total hits. Despite the lower total numbers of sequences, the *Geobacter* and *Desulfovibrio* genera were both detected in all the surface brine metagenomes in similar proportions to the deep subsurface brines.

The functional profiles are largely similar between the surface and subsurface environments. Before comparing the functional profile, the data was normalised between the subsurface and surface environments using the DESeq package (Anders and Huber 2010) built into MG-RAST. This was found to be effective in normalising read counts in sequencing data (Lin et al. 2016).

Complete pathways and key genes related to denitrification were only found in the subsurface metagenomes. Some hits for a number of genes involved in denitrification (EC 1.7.2.1 and 1.7.99.7) were observed in the South Bay Salt Works metagenomes (4440433.3 and 4440430.3), but not the entire pathway unlike in the subsurface (see Figure 5.8). Similar pattern was observed in the dissimilatory nitrate reduction process, with key genes only being identified in the South Bay Salt Works metagenomes (4440430.3). In contrast, key enzymes involved in assimilatory nitrate reduction such as ferredoxin-nitrite reductase (EC 1.7.7.1), were more abundant in the surface metagenomes. Further sequencing in the surface brines may have revealed minor denitrification or dissimilatory nitrate reduction capabilities,

but based on the currently available normalised data sets, it appears they are more abundant in the deep subsurface.



**Figure 5.8.** Heatmap of subsystems functional genes coding for enzymes involved in denitrification in the Boulby, Bras del Port and South Bay Salt Works metagenomes. Data has been normalised using the DESeq package built into MG-RAST. Unlike in the surface brines, denitrification pathways are complete and genes proportionally more abundant in the deep subsurface Boulby brines.

The ability to carry out denitrification has been identified in a small number of Halobacteriaceae in the *Haloarcula* (*H. marismortui* and *H. vallismortis*), *Haloferax* (*H. denitrificans* and *H. mediterranei*) and *Halogeometricum* (*H. borinquense*) (Oren 2013) genera. It is possible that the ability to deal with the lower oxygen levels in the deep

subsurface using methods like denitrification, especially if nitrate is available as recorded in 44 XC, could lead to certain genera in the Halobacteriaceae family being better represented in hypersaline deep subsurface environments in comparison to those at the surface.

Like the deep subsurface metagenomes, sulphur cycle was largely incomplete in the surface brines examined, as has been document previously (Fernández et al. 2015), with genes coding for adenylyl-sulfate reductase (EC 1.8.99.2) and dissimilatory sulfite reductase (EC 1.8.99.5) being absent.

Some evidence for arginine fermentation was seen. Small numbers of hits were found in a few of the South Bay Salt Works metagenomes relating to arginine deiminase (EC 3.5.3.6), carbamate kinase (EC 2.7.2.2), catabolic ornithine transcarbamylase (EC 2.1.3.3) in one of the South Bay Salt Works (4440438.3) and partially complete in a number of others (4440433.3, 4440430.3, 4440429.3). Some evidence for ethanol fermentation also exists in the Bras del Port and Salt Bay Works metagenomes and homolactic fermentation in the Salt Bay Works metagenomes. Fumarate reductase (frdABCD EC 1.3.5.4) was also present in all the Bras del Port metagenomes, but TMAO reductase (EC 1.7.2.3) was absent from all metagenomes, aside from a handful of hits in 215.

Like the deep subsurface brines, the Salt Bay Works metagenomes had hits related to endoglucanase (EC 3.2.1.4) and beta-glucosidase (EC 3.2.1.21), both of which are involved in the degradation of cellulose. The surface and deep subsurface brines also both displayed incomplete pathways or key genes were missing from methanogenesis, methanotrophic and hydrocarbon pathways. RuBisCo was present in a number of the South Bay Salt Works metagenomes (4440438.3, 4440429.3), as in the Boulby brines. A number of enzymes involved in the reverse TCA cycle were found in the surface metagenomes, such as citrate synthase (EC 2.3.3.1) and 2-oxoglutarate:ferredoxin oxidoreductase (EC 1.2.7.3), but pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and ATP citrate lyase (EC 2.3.3.8) were absent from all surface metagenomes. The Wood-Ljungdahl pathway was incomplete in both surface and subsurface brines.

Overall, the taxonomic and functional pathways were relatively similar between the surface and subsurface. Taxonomically they are very similar, with Halobacteriaceae dominating. Evidence of cellulose degradation and fermentation were common to both environments, and little evidence of genes related to hydrocarbon degradation, methanogenesis, methanotrophy and dissimilatory sulphate reduction were present in either. Denitrification could offer a



potential difference between the two, and may be more important in the deep subsurface, potentially due to the lack of oxygen.

## 5.3 Limitations

The metagenomics techniques used here do not give any information on whether certain organisms are active or inactive. It is only possible to see which pathways and organisms are present and then make reasoned arguments for their activity/inactivity in the brines based on the current literature.

It is also not possible to assume that all pathways and organisms are well characterised. Pathways related to hydrocarbon degradation in halophiles are very poorly characterised for example (Fathepure 2015), and may therefore not be detected in the analysis. Some genes coding for metabolic pathways may also be present in the environment, but in low abundance which further sequencing would detect. In this way, metagenomics allows us to identify the presence of certain pathways and organisms to a reasonable degree of accuracy and estimate their importance, but their absence is not definitive.

Contamination also presents a limitation to this work. Contamination can occur at any stage of the experimental process, including during collection, DNA extraction and sequencing from any of the reagents used. Although blanks were run through all the processes and then failed to produce PCR products and sequences, this does not necessarily mean that contamination is absent from metagenomes. Illumina chemistry works best when there is an abundance of DNA. Low concentrations of DNA may not sequence successfully, but that same DNA will sequence when mixed with higher concentrations of other DNA. This is why when running low biomass samples, sequencing centres will occasionally run multiple samples together and separate them after with barcoding. Consequently, although our blank failed to sequence, it may have been able to produce sequences if run with other DNA. However, these would have been extremely low numbers of sequences that would not impacted the main results.

Another aspect worth noting is that Boulby has been open for over 40 years. Some human contamination is inevitable in an active working mine. Two of our brines sequenced here (44 XC and 215) were sampled directly from or very close to wall seeps so contamination from

the mine air would have been minimal. Some contamination may have come from the pumps, but would have been extremely low since all had been in place for at least a year and have constantly had brine flowing through them. This means any initial contamination should have been diluted down to close to undetectable levels. As a brine pool, Billingham would have been more susceptible to mine air contamination, although the brine did not appear to have a particularly long residence time in the pool due to it being constantly pumped out, meaning the community may not have much time to significantly change. Even if it had a long residence time, the community present is generally compositionally very similar to 215 and 44 XC, suggesting it is probably representative of brines in the deep subsurface.

Despite the controls and precautions taken, contamination issues can only be mitigated rather than completely avoided, especially given the active mine context. However, based on the discussion above, it is not thought that they impacted the conclusions or results of this study in any significant way.

## 5.4. Conclusion

The DNA was extracted successfully and used to sequence three metagenomes from the deep subsurface brines 44 XC, 215 and Billingham Baths. Using these metagenomes, the taxonomic and functional profile of the community was examined. The results of this work produced the following conclusions:

- Extracting DNA from the brines found in Boulby with high clay content can be achieved by adding a phosphate buffer prior to cell lysis.
- Even after using a variety of DNA extraction techniques and around 10 L of brine, DNA could not be recovered from brines 101-P and 29 XC. This consolidates the results of Chapter 4 in relation to 101-P being uninhabitable, and gave a possible explanation (additional stress from pressure) for the lack of organisms present in 29 XC, despite it being habitable (see Chapter 4 for more details).
- The three deep subsurface brines are relatively similar in both taxonomic and functional profiles. As discussed in Chapter 3, all the three came from the Sherwood Sandstone aquifer, but have taken different pathways through the subsurface before

being intersected by the mine. Despite this, the communities in all three deep subsurface brines were dominated by the family Halobacteriaceae and generally the three brines contained very similar organisms.

- Functional pathways were also largely consistent between the three deep subsurface brines, with pathways related to the metabolism of a range of simple monosaccharide, polysaccharides and amino acids abundant, including cellulose degradation pathways. Functional pathways related to various more complex carbon sources such as methane, PAH's and other hydrocarbons were largely incomplete. Viable autotrophic pathways are missing key functional genes. Gene's related to fermentation and denitrification appear to be viable means of energy acquisition when the oxygen levels are low.
- At higher taxonomic levels, the surface brines were very similar to their deep subsurface counterparts. At lower levels, certain genera within the dominant Halobacteriaceae family were represented differently, for example *Haloarcula* was proportionally more common across the three deep subsurface brines than in the surface brines. The carbon usage patterns were also relatively similar, with a range of simple monosaccharide, polysaccharides and amino acids, including cellulose degradation pathways being present in all, but genes missing for various hydrocarbon degradation and autotrophic pathways. RuBisCo's metabolic signature is retained in the subsurface metagenomes, despite the differences in light levels in the two environments. Both the surface and subsurface showed fermentation and dissimilatory fumarate reduction capabilities, but denitrification pathways were only detected in the deep subsurface examples. The sulphur cycle was incomplete in all surface and subsurface brines.
- Generally, these results indicate that at NaCl levels close to saturation, NaCl is the dominant influence on brine microbial community structure, regardless of the environmental differences between the deep subsurface and surface.

Further work should aim to sample a greater range of brines from the deep subsurface. Attempts should be made to extract DNA from a greater range of geochemically diverse brines and compare them to surface and other deep subsurface brines. The brines sequenced here displayed relatively similar geochemical profiles, so increasing geochemical diversity would be an excellent way to understand the variability of communities living deep subsurface brines, particularly brines dominated by anions such as sulphate. Work also needs to be done on better identifying the pathways used by halophiles to metabolise hydrocarbons.

## Chapter 6: Can diverse carbon sources drive the deep evaporite biosphere?

The work presented in Chapter 5 shows that the communities present within the brines at Boulby are dominated by organoheterotrophs. For these communities to survive within deep subsurface evaporite deposits, it is necessary for them to have access to carbon sources for both energy and synthesising organic compounds for growth. This chapter aims to examine the carbon usage capabilities of the organisms in the mine and to determine if any *in-situ* carbon sources can be utilised by the organisms present for growth. To achieve this, a number of questions are investigated:

- i. Are there any carbon sources in the salt rocks from the mine that can be utilised by the heterotrophs for growth? Similar salt rocks have previously been shown to contain cellulose fibres (Griffith et al. 2008) and it has already been demonstrated in Chapter 3 that gaseous hydrocarbons exist within the sylvinite salt rock in the mine. Certain salt rocks may therefore be able to supply the communities present in the brines with carbon.
- ii. Do oil seeps encountered in the mine promote the growth of halophilic communities? Oil seeps are regularly encountered in several locations around the mine. These could provide a carbon source to the halophiles living in the Zechstein sequence.
- iii. Can the microbial communities present in the brines utilise a diversity of laboratory grade carbon compounds in aerobic and anaerobic conditions? Given the low organic carbon contents of the deep subsurface and the energetic demands of living at high salinities, understanding the flexibility of the community in the brines to use a range of carbon sources is important for understanding how they deal with long-term survival in these deep subsurface hypersaline environments.

Enrichment techniques were used to answer these questions. To examine the usability of any carbon sources present in the salt rocks, samples were collected from the mine and added to mineral media to determine if any carbon compounds present promoted growth. This involved the careful design of several experiments to control for the impact on growth of any geochemical changes made by the addition of the salt. Growth was also examined using a

range of laboratory grade carbon sources and natural oil seeps collected from the mine. DNA was then extracted from the enrichment cultures and the community examined to see what organisms could utilise the various carbon sources tested.

## **6.1 Methods**

### **6.1.1 Sampling techniques**

Brines sampled for culturing were collected with the same methodology as detailed in Section 4.1.1, although the anaerobic samples did not have cysteine added. Salt rocks were collected at the same time as those detailed in Chapter 3. These were taken from fresh surfaces using a sterile rock hammer to minimise the carbon contamination on to the surface of the solid salt. Lumps of salt rock were transported in furnaceed tin foil and then wrapped in sterile plastic bags and placed in a cooler with freezer packs. Once transported back to the University of Edinburgh, the salt was frozen in the -80 °C freezer to preserve any carbon compounds present. Mine geologists employed by Israel Chemicals Ltd (ICL), sampled the oil seeps using polypropylene bottles. The oil seeps were found in the halite layer of the mine, just below the sylvinite seam and were accompanied by a methane rich gas seep.

### **6.1.2 Ancient carbon trapped in Permian salt rocks**

Enrichment techniques were used to investigate whether any carbon trapped in the salt rocks could promote growth in halophiles from the mine. This involved incorporating salt minerals taken from the mine into the media without the addition of any other carbon sources to determine if the addition of these salt rocks promoted growth above several controls.

#### **6.1.2.1 Mineral media preparation**

A basic mineral media was devised which contained compounds aiming to provide the basic elements required for the growth of halophiles (Table 6.1). The idea was to exclude carbon sources from the media and test whether by including the salt rocks, growth could be stimulated. Due to the high levels of dissolved salts in this mineral media, adding a variety of

trace elements was considered unnecessary (e.g. Schneegurt 2012). All the CHNOPS elements are present in media in various forms. Nitrate and sulphate sources were added to provide an alternative electron acceptor to oxygen. As far as possible, alternative electron donors to carbon were excluded from the media. NaCl was added in concentrations to create an environment more representative of that in the brines, but not at the saturation levels measured in the brines. This was due to persistent issues experienced with media crystallisation and reduced growth rates. This mineral media was also used to test growth on laboratory grade carbon sources and hydrocarbons, and did include the addition of some trace elements when used for anaerobic media (see Section 6.1.3).

Solution	Chemical	Quantity
1	NaCl	250.0 g
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	20.0 g
	KCl	2.0 g
	FeCl <sub>3</sub>	0.1 g
	dH <sub>2</sub> O	989.5 ml
2 (add 10 ml to part 1)	NaH <sub>2</sub> PO <sub>4</sub>	2.6 g
	Na <sub>2</sub> HPO <sub>4</sub>	20.176 g
	dH <sub>2</sub> O	100.0 ml
3 (add 0.5 ml to part 1)	NaNO <sub>3</sub>	5.0 g
	dH <sub>2</sub> O	50.0 ml

**Table 6.1.** Mineral media used to examine carbon usage. Depending on the experimental set-up, part 1 was either autoclaved or filter sterilised. Parts 2 and 3 were always filter sterilised and added to part 1 in a laminar flow-hood. 0.5 ml of part 2 and 2.5 ml of part 3 were added to part 1 to the final volume 1 litre, giving NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub> and NaNO<sub>3</sub> final concentrations of 0.026 g/l, 0.2176 g/l and 0.5 g/l respectively. These quantities were based on those typically used to grow halophiles (see Schneegurt 2012). The pH was adjusted to 7.5 using filter sterilised sodium hydroxide and hydrochloric acid.

To examine how the addition of the salt rocks from the mine impacted growth, several different versions of this mineral media were used. In Experiment 1 (Figure 6.1), two additional versions of the media were made up with 50 g of NaCl in part 1 replaced with 50

g of halite and sylvinite collected from Boulby mine. The basic mineral media was used as baseline control for growth. Additional media types to control for specific factors were then added for the full experiment (discussed below in Section 6.1.2.4).

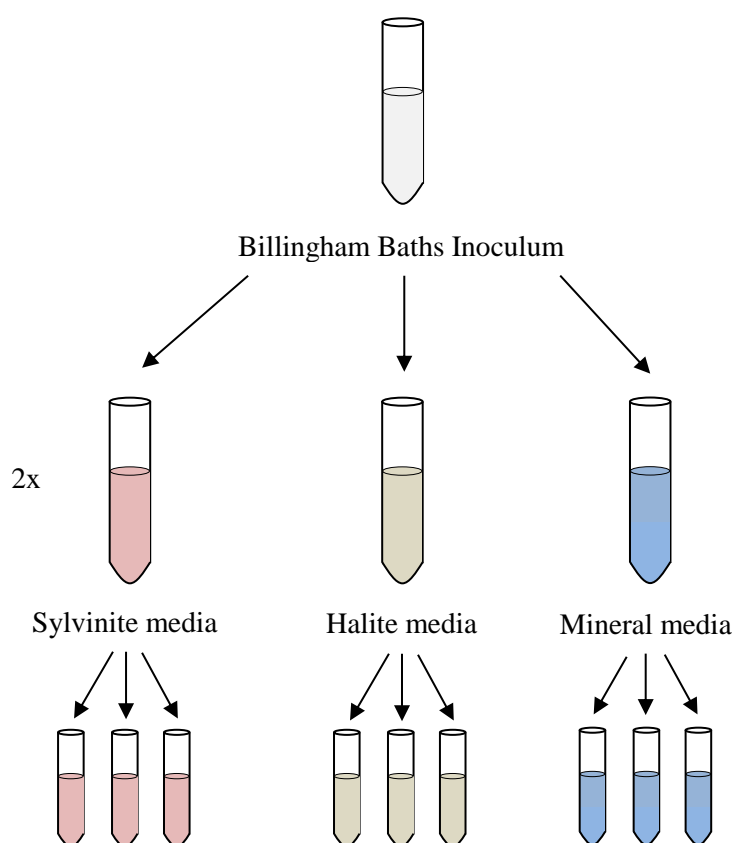
Autoclaving this mineral media and its variants provided a dilemma, particularly in relation to the media containing the dissolved salt rocks. Not autoclaving them meant the salt media had to undergo filter sterilisation prior to inoculation. This was problematic since much of the detrital material found in the sylvinite and halite, which may have included valuable organic matter, would be lost. Autoclaving, however, would result in the alteration or loss of some of the contained organics. With neither method being perfect, it was decided that losing the detrital material was too valuable for the experiment so the majority of the work took place with the autoclaving method. However, to test its impact, one experimental run (Experiment C) was carried out with filter sterilised salt media. The media was made up using the same recipe as above. Part 1 was allowed to settle for an hour to reduce filter clogging and then filter sterilised through a 0.45 µm filter followed by a 0.22 µm filter.

Although it was inevitable that some small amounts of carbon would be present in the reagents used, steps were taken to reduce the amount of carbon present in the mineral media, aside from that contained in the halite and sylvinite, in an effort to reduce that chance that any additional growth was masked. Plastic equipment such as universal tubes were therefore avoided and furnace (450 °C for 6 hours) glassware used instead to culture organisms. Due to the number of replicates required for the experiment, it was not possible to use normal glass conical flasks for the enrichments. Instead 15 ml glass test tubes were used. After being furnace, these tubes were autoclaved for 20 minutes at 121 °C covered with a foam bung wrapped with tin foil. After cooling, 5 ml of the media was added to each test tube. The pH of each media was checked again after autoclaving before the start of the experiment to ensure it was consistent and would not impact growth. The tin foil was then removed and each test tube sealed with plastic paraffin film over the foam bung to minimise water loss and prevent crystallisation.

### **6.1.2.2 Inoculation**

The main and preliminary experiments were carried out using a small number of different inocula. Samples of Billingham Baths were added to the three types of media (mineral media and mineral media with halite and sylvinite) and grown for 45 days and then transferred and left for another 45 days. This was to dilute out any organics present in the original

environmental sample, which may have influenced growth and masked the impact of the carbon from the halite and sylvinite. After the second 45-day period, a 100  $\mu$ l aliquot was taken out of each and used to start the corresponding enrichment for the preliminary experiment. For the rest of the experiments, an aliquot of each of the three media types was used to inoculate a greater range of media types (see Figure 6.3). Instead of using an isolate for these experiments, it was decided that a complex community better emulated the natural conditions found in the brines.



**Figure 6.1.** Experiment 1 set-up. Billingham baths brine was used to inoculate enrichment cultures which were grown up and transferred once before the start of the preliminary experiment. Growth measurements using agar plates were carried out in triplicate on each test tube. This work was used to determine if the addition of these salt minerals to the media changed growth rate and cell density.

Once inoculated, the media was placed in a 37 °C shaker incubator and growth monitored weekly. The shaker incubator had its windows blacked out with tin foil to keep the test tubes in the dark which would better emulate the subsurface environment. The preliminary experiment (1) lasted 21 days, and the main experiments (A, B and C) 54 days. The test



tubes are not as effective as the glass conical flasks at preventing oxygen stratification. This was mitigated by using 5 ml of media in a 15 ml tube, meaning the shaker was more effective at constantly disturbing the media. In addition to this, the test tubes were manually removed and disturbed every two days to encourage constant oxygen movement to the lower parts of the tubes. Monitoring growth in these test tubes during the experiments is discussed below.

### 6.1.2.3 Monitoring growth

In the halite and sylvinite media, monitoring growth proved to be challenging. Since monitoring growth was critical to the experiment, a number of different methods were trialled. Optical density (OD) was found to be unsuitable for several reasons. The cell densities experienced in some of the trial experiments resulted in very low cell densities in the media, making OD, a method more effective at high cell concentrations, a poor way to determine growth. Worse still, some of the halite and sylvinite media were full of opaque detrital matter such as clays. Attempts to remove them by centrifugation or filtering always resulted in the loss of cells making the method unfit for determining growth within this experiment.

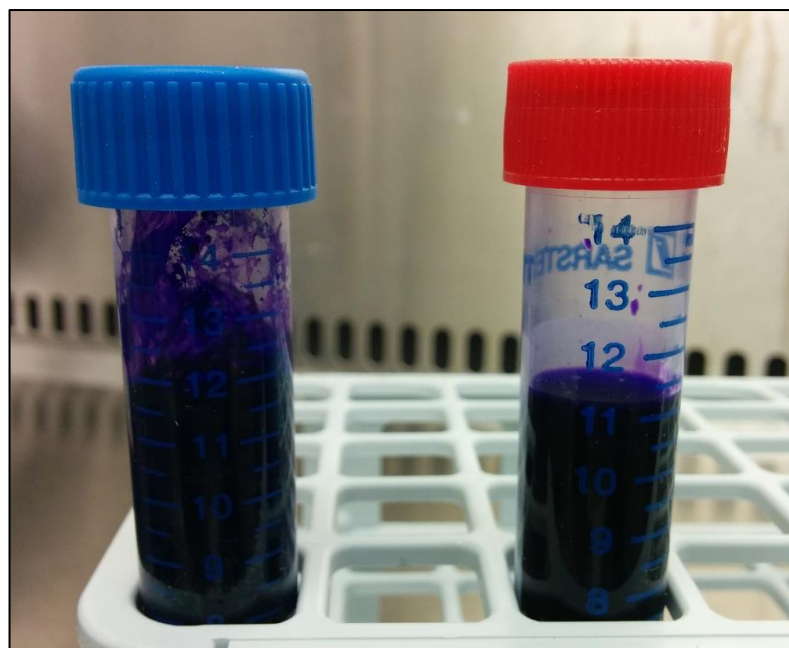
Attempts to conduct microscopic counts suffered similar issues. A variety of stains including DAPI, SYBR Gold/Green and Acridine Orange were all trialled. Cells could be visualised, but achieving reliable counts was very difficult due to the clay minerals and other detrital matter. Not only did these visually mask cells, but they also induced auto-fluorescence. Sonicating the cells off the clay minerals was considered as an option, but concerns about lysing them prevented this method from being employed.

Protein assays such as the Bradford assay were also considered and proved unsuitable. CFU's were measured at a maximum in the different media of  $\sim 3 \times 10^4$  per ml which, assuming equivalent *Escherichia coli* protein contents per cell, equates to around 0.00465  $\mu\text{g/ml}$  of protein. This is several orders of magnitude below the minimum 0.5  $\mu\text{g/ml}$  required by a traditional Bradford assay. It may have been possible to quantify protein concentration whilst the enrichments were at their most dense using more sensitive versions (e.g. Zor and Selinger 1996), but this would have still meant a lack of resolution at lower concentrations. Concentrating cells down to the required cell densities and then back calculating would

require excessively large cultures which were impractical given the laboratory set-up available.

DNA extractions also proved unsuitable for monitoring growth in the cultures. As shown in Chapter 4, the media contained clays from the dissolved sylvinite and halite. These presented a problem due to the difficulties associated with extracting DNA from clay rich environments. To achieve DNA extraction, it was necessary to run the media through a clay proof protocol as detailed in Chapter 5. Faster methods were unable to successfully recover DNA. Overall this resulted in a DNA procedure that was both far too slow and too expensive to be used as a realistic method of doing triplicate time points in a growth curve for each media type.

Crystal violet stain, which often used to quantify growth in biofilms (e.g. Li et al. 2003, Childers et al. 2013), was also trialled as a method to quantify growth. This method typically involves using crystal violet to stain cells, after which excess crystal violet is washed away with PBS and the cells lysed with acetic acid or ethanol. The amount of crystal violet released is then measured using a spectrophotometer. To avoid lysing the halophilic cells in the dyeing and washing phases through osmotic shock, PBS with 25 % NaCl was used to wash the cells and 25 % NaCl added to the crystal violet stain. Unfortunately, the high salt concentration reacted with the crystal violet mixture and components began to separate out (see Figure 6.2) making the stain unusable. When not using NaCl, no difference was observed between the blank and culture media, suggesting either that the cell density was too low to detect with the crystal violet, or the cells lysed when exposed to the low salinity crystal violet solution. Another issue arose from the absorbance of crystal violet by the detrital matter in the media, which would have swamped any signal from the crystal violet released from the cells post lysis due to the low cell densities.



**Figure 6.2.** Crystal violet separation observed when mixed with NaCl. The dye began to separate when 25 % NaCl was added (tube on the left) to prevent early cell lysis. The tube on the right shows how the dye appears without the addition of NaCl.

After trialling these methods, it was decided the best way to monitor growth would be to use nutrient agar plates. Although inherently limited in their inability to pick up cells unable to grow on agar plates, they can detect low cell densities and were not impacted by the high clay content of the media.

Agar plates were made up by using a simple nutrient agar (Sigma Aldrich, Missouri, USA) with the addition of 250 g/l NaCl and 1 g/l KCl. pH was balanced to 7.5 using sodium hydroxide and hydrochloric acid. It was found that with this higher salt content, an additional 5 g/l of agar (Agar No. 1 Oxoid) had to be added to ensure the plates set properly and were firm enough for spreading. This mixture was then autoclaved at 121 °C for 20 minutes and poured into petri dishes in a laminar flow-hood. A time point measurement consisted of taking the glass test tubes, opening them in a laminar flow-hood, mixing the contents to evenly distribute cells and removing a 25 µl aliquot with a pipette and dropping it onto a plate. Plates were divided into three sections, one for each 25 µl aliquot from each test tube every time point, making a triplicate measurement. A sterile glass spreader was then used to spread the media on the plate, and plate left to dry in the flow-hood. Once dry, all the plates were wrapped with paraffin film and placed inside sterile bags to prevent the loss of moisture and the formation of crystals on the plates during incubation. Although not tightly sealed,

these bags were opened twice a week to ensure oxygen was being circulated. Plates were incubated at 37 °C until colony size was countable, normally taking around 4-6 weeks.

#### **6.1.2.4 Control development**

To get an accurate idea of whether a carbon source present within the solid salt could promote growth in halophiles from the mine, several different controls were added to the experimental design after the preliminary experiment. This included three additional media conditions to control for specific factors. Triplicate blanks consisting of the media with no inoculum were also included for each media type.

The first control consisted of the mineral media detailed previously Section 6.1.2.1. This control was designed to capture and quantify any baseline level of community growth without the addition of any salt rocks to the media. After Experiment 1 showed that growth was faster and to a higher cell density in the sylvinite media (see Figure 6.4), several other controls were added to the experimental set-up (Experiments A, B, C) to see if this additional growth was caused by the shift in media ionic composition from the dissolution of the salt minerals, or from any carbon contained within the salt minerals. The second control media was therefore designed to artificially recreate the geochemistry introduced to the media by the dissolving the salt rocks, but without the additional carbon source, using laboratory grade reagents. Creating this control involved a number of stages (see Section 6.1.2.3). Mineral media with the additional halite and sylvinite was filtered through 0.45 µm syringe-driven polycarbonate filter units (Merck Millipore, Massachusetts, USA) to remove insoluble material. This fluid then underwent IEC and ICP-OES analysis using the same methods described in Section 3.2.2.1, allowing the exact ionic composition of the media, once the salt rocks have been dissolved, to be determined. New recipes were then created to match those detected by the ICP-OES and IEC (Table 6.3) to control for how changes in geochemical composition induced by the dissolution of the salts impacted growth. Some residual difference between the compositions measured in the halite and sylvinite media and the recipes created to copy them were present (see Table 6.2). The largest discrepancy between the measured values and the media recipes devised being in the Na and Cl levels. These differences were kept under 1 g/l. With the quantity of ions present in the media being so high, it was assumed that these minor discrepancies would not have a notable impact on the growth conditions and therefore would not damage the usefulness of the media as a control.

	Halite Media			Sylvinite Media			Base Mineral Media
Ions	Ions measured in salt rock media	Ions provided by new media	Difference	Ions measured in salt rock media	Ions provided by new media	Difference	Ions measured
K	2.276	2.276	0.000	13.356	13.356	0.000	2.350
Na	98.112	98.612	0.500	85.354	84.666	-0.688	104.839
Mg	0.125	0.228	0.103	0.132	0.132	0.000	0.130
Fe	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ca	0.003	0.003	0.000	0.008	0.008	0.000	0.000
Cl	154.190	154.412	0.222	139.582	139.710	0.128	157.792
NO <sub>3</sub>	0.779	0.779	0.000	0.618	0.618	0.000	0.354
SO <sub>4</sub>	9.287	9.287	0.000	7.496	7.496	0.000	8.084
PO <sub>4</sub>	0.065	0.065	0.000	0.055	0.055	0.000	0.055

**Table 6.2.** Comparison of ionic composition of natural salt rock and artificial media (in g/l).

It is not possible to get an exact balance of ions measured in the salt rock media and those provided by the new artificial media, but the differences between the two were kept under 1 g/l. The table also displays the ions measured in the base mineral media recipe outlined in Table 6.3. The addition of halite and sylvinite clearly changed the concentration of many ions, including K, SO<sub>4</sub> and NO<sub>3</sub>.

	<b>Artificial sylvinite media</b>	<b>Artificial halite media</b>
<b>NaCl</b>	210.404	249.937
<b>KCl</b>	25.383	4.241
<b>CaCl<sub>2</sub></b>	0.022	0.009
<b>K<sub>2</sub>HPO<sub>4</sub></b>	0.100	0.119
<b>NaNO<sub>3</sub></b>	0.847	1.068
<b>FeCl<sub>3</sub>.6H<sub>2</sub>O</b>	0.000	0.000
<b>NaSO<sub>4</sub></b>	10.445	10.445
<b>MgSO<sub>4</sub>.7H<sub>2</sub>O</b>	0.593	1.069

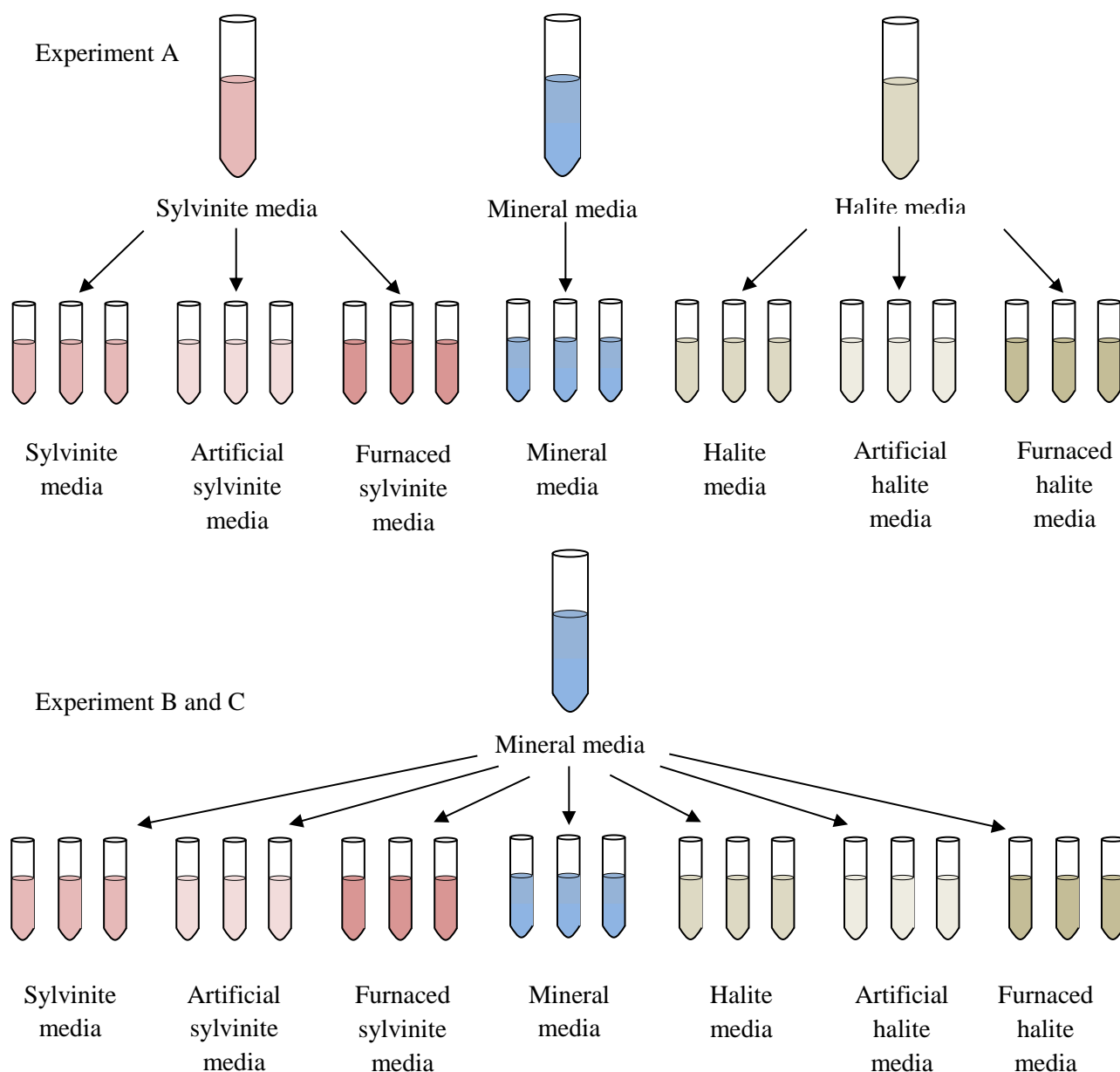
**Table 6.3.** Recipes for media (in g/l) used to control for changes in brine geochemistry to determine the impact of any carbon introduced by the salt rock. These were devised from the ICP-OES and IEC results detailed in Table 6.2.

As discussed in Chapter 3, the sylvinite, and halite to a less extent, contain clay minerals and other insoluble material. These might provide surfaces for the microorganisms in the media to grow on and therefore impact growth rate or the measurement of growth. This aspect could not be incorporated into the synthetic versions of the halite and sylvinite media. Additionally, there may have been a small number of ions present in the natural salt rocks that were not picked up by the ICP-OES and IC. For these reasons, a third media variant made with furnaced halite and sylvinite was added to control for these factors.

To create this third control, halite and sylvinite were both taken and ground down with a mortar and pestle into a fine power. This was to allow any carbon compounds trapped in the salt to be vaporised and escape. The powder was then weighed into 50 g portions and wrapped in tin foil and baked at 450 °C for 6 hours. The salt was weighed out prior to furnacing to ensure that any water or carbon loss did not result in the additions of excess salt to the media. After it had cooled, the salt was added to the mineral media in the same fashion as the un-furnaced halite and sylvinite above.

#### **6.1.2.5 Main experimental design (Experiments A, B and C)**

The main experimental set-up incorporated the additional controls described above, as is shown on Figure 6.3. It also varied the starting enrichment used to initiate the experiment more than the preliminary work, using cultures grown up from Billingham baths in halite, sylvinite and mineral media. Additionally, it also used the enrichment grown in the mineral media to start all the media varieties (see Figure 6.3). This was to control for any variability across the communities grown in different media.



**Figure 6.3.** Main experimental set-up with all controls. The basic experimental set-up was the same as Experiment 1. However, a wider range of controls were incorporated into the design. Growth was again monitored for every replicate using triplicate agar plate measurements. Blanks are not shown. In Experiment A three different enrichment cultures were used as inoculum. Experiment B and C used one inoculum for all replicates. All inoculum cultures were started from Billingham Baths brine as is shown on Figure 6.1, but with one additional transfer of the starting media over the preliminary experiment required to maintain the cultures.



### 6.1.3 Community carbon utilisation capabilities

In order to get a first look at the carbon usage abilities of the community in the deep subsurface evaporite brines and the wider Zechstein deposit, enrichment techniques were used to examine growth on a number of refined laboratory grade carbon sources and natural oil seeps collected from the mine. This aimed to help determine which carbon sources could be supplying the brine communities.

After collection, the two oil seeps were filtered through a 0.22 µm filter and frozen in 15 ml universal tubes to preserve them. Briefly, two types were collected, a rich orange-yellow coloured semi-fluid (2001 salt road) and a dark brown viscous fraction (L1026). ICL kindly provided some commercial work commissioned specifically to analyse the composition of the hydrocarbon seeps entering the mine (Table 6.4). The two hydrocarbon seeps have quite different compositions. L1026 is the heavier fraction, containing higher proportions of higher molecular weight hydrocarbons such as aromatics and polyaromatics. 2001 salt road contains more alkanes and has a higher hydrocarbon to non-hydrocarbon ratio. We did not observe the formation of an aqueous phase in either of the oil samples used in this work.

	MPLC & asphaltene precipitation data					
	Fraction (wt% of EOM/oil)				Ratios	
Sample	Alks.	Aroms.	Pols.	Asph.	Alks/ aroms	HC/ non-HC
2001 salt road	86.8	9.5	3.3	0.4	9.14	26.03
L1026	39.2	43.3	17.2	0.3	0.91	4.71

**Table 6.4.** Composition of the two oil seeps used in this work collected from Boulby Mine. These data are shown here with the permission of ICL and are taken from a private commercial analysis. EOM = Extractable organic material. Data was collected using Medium Pressure Liquid Chromatography (MPLC).

These results were obtained by quantitative triple fraction Medium Pressure Liquid Chromatography (MPLC) at the APT AS laboratories in Kjeller, Norway. The methods used to obtain the data in Table 6.4 are as follows. Asphaltenes were firstly removed by adding

pentane at 40 times the volume of the natural oil, and the mixture stored for 12 hours in the dark before being centrifuged and filtered, and the asphaltenes weighed. MPLC was then carried out on the samples using methods described in Radke et al. (1980). The system included a sample injector and collector, two HPLC pumps and two packed columns. Kieselgel 100 was used to fill the pre-column and then heated to 600 °C for 2 hours. A LiChroprep Si60 was used as the main column. This was heated at 120 °C for 2 hours with a helium flow to remove any water. Approximately 30 mg of the deasphalted oil was then diluted in 1 ml of hexane and injected into a sample loop. The saturates were collected by flushing hexane through the main column, pre-column and sample loop. Aromatics were then removed by back flushing the main column again with hexane. Finally, polars were collected by flushing dichloromethane through the pre-column. All the solvents were then removed using a Turbovap unit, transferred to small vials, dried and each fraction weighed.

As mentioned, in addition to these oil seeps, enrichments were set up using a number of laboratory grade carbon sources selected to test the ability of the brine microbial communities to utilise a small range of carbon molecules across aerobic and anaerobic conditions. These carbon sources were: yeast extract, glucose, trisodium citrate and xylan, which together provide some molecular diversity. Yeast extract contains a rich mixture of carbon sources. Glucose is a simple sugar and trisodium citrate a simple acid. Xylan is a long chain polysaccharide and provides a more recalcitrant source of carbon for the organisms in the brines.

Enrichments for all carbon sources were set up in triplicate. Brine 215 and Billingham Baths (see Chapter 3 for details) were used as inocula since these were the only two brines available at the time the experiments were attempted. Aerobic carbon cultures were set up in the same way as described in Section 6.1.2.1/6.2.1.2, using furnaced glass test tubes. The basic mineral medium used was the same as described in Section 6.1.2.1. This media was seen to provide the necessary essentials for the growth of halophiles. Yeast extract and xylan and were added to the minimal media in a concentration of 10 g/l and then autoclaved. Glucose and trisodium citrate were both added to 50 ml of liquid, filter sterilised and then added to the mineral media after it was autoclaved at the same concentrations as the yeast and xylan. Similarly, the hydrocarbons were filter sterilised and added to the mineral media after autoclaving at concentrations of 1 ml per 5 ml of mineral media.

Anaerobic carbon enrichments were examined using the same set-up as described in Section 4.1.2.1 with a couple of small modifications. The serum bottles were furnaced prior to the

introduction of the mineral media to reduce the amount of carbon present. The same mineral media used in the aerobic was used for the anaerobic media, but sparged with N<sub>2</sub>. A trace element solution (DSMZ SL 6) was added to part 1 (10 ml/L) of the mineral media which contained MnSO<sub>4</sub>·H<sub>2</sub>O (0.5), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.1), CoSO<sub>4</sub>·7H<sub>2</sub>O (0.18 g), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.18), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.01), KAl(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O (0.02), H<sub>3</sub>BO<sub>3</sub> (0.01), NaMoO<sub>4</sub>·2H<sub>2</sub>O (0.01), NiCl<sub>2</sub>·6H<sub>2</sub>O (0.03) and Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O (0.03 mg/l) (all g/l unless specified otherwise). Nitrilotriacetic acid, MgSO<sub>4</sub>, FeSO<sub>4</sub> and NaCl were not included since MgSO<sub>4</sub>, FeSO<sub>4</sub> and NaCl were already present in the base mineral media and nitrilotriacetic acid was a carbon source. The trace element solution was stored at 4 °C in the dark and pH balanced to 7.5. The filter sterilised oil seeps, glucose and trisodium citrate solutions (same concentrations as the aerobic cultures) were added to sterile 10 ml serum bottles and topped with a sterile bung and crimp sealed. Two needles were then inserted through the bung and the bottles sparged for 10 minutes using N<sub>2</sub> gas. They were then injected into the autoclaved mineral media. Media parts 2 and 3 were set up in the same way. pH was checked once all the parts had been mixed in each individual serum bottle and balanced if required with sterile anaerobic HCl and NaOH. Growth in the oil seep and the other carbon sources was examined using the same microscopy techniques outlined in Section 4.1.2.1.

Cysteine and resazurin were not included in the carbon cultures, since they contain carbon. Hydrogen sulphide was considered as an alternative, but due to the desire to avoid providing alternative electron donors to the carbon sources present (a problem with all reducing agents), it was not used. The lack of a reducing agent means the cultures would have likely retained trace amounts of oxygen despite the long sparging times.

It is worth noting that to determine which fraction of the oil seeps were being used by the brine communities for growth, hydrocarbon structure/concentration would need to be monitored during enrichment. Here we are testing only to see if the oil seeps promote growth. Further work could focus on which part of the seep is being used.

### **6.1.4 Amplicon sequencing**

DNA was extracted from the cultures and underwent PCR amplification using the same methods as described in Chapter 5. A number of cultures from the aerobic and anaerobic carbon cultures were sent for amplicon sequencing. DNA extraction methods were the same as those detailed in Chapter 5 (PowerMax® Soil DNA Isolation Kit). All amplicon

sequences were generated using Illumina MiSeq technology at Research and Testing, Texas, USA. Some initial data processing was done by Research and Testing (detailed below). Their diversity analysis was not used in this work since it resulted in a lot of unassigned sequences. Consequently, several different microbial analysis techniques were used to ensure an accurate representation of the community was generated. The FASTA data files from Research and Testing were the starting point for the analysis.

Research and Testing carried out a number of processes to denoise and check the output files for chimeras, which aims to correct read errors in next-gen sequencing. Denoising aims to correct read errors that infrequently occur in Illumina and other next gen sequencing methods (>99.6% error rate for Illumina) (Huse et al. 2007, Quince et al. 2011, Quail et al. 2012). Due to the very large number of reads, it is necessary to remove these errors to improve the quality of the output. Research and Testing's pipeline involves merging the forward and reverse reads and running them through a proprietary quality trimming algorithm. This output then undergoes prefix dereplication, with singleton clusters remaining in the output (although these were removed further down the analysis pipeline). Clustering and OTU selection was then carried out on the dataset using UPARSE and USEARCH respectively. OTU's were then checked for chimera's using UCHIME in de novo mode, and any chimeric sequences removed.

After receiving the data from Research and Testing, all the further processing took place in QIIME (Caporaso *et al.*, 2010). Following mapping file validation, the FASTA files provided by Research and Testing were combined with the sequence quality scores into a FASTQ file and the barcodes and reads separated into two files so additional quality control could be carried out. To reduce the influence of sequencing errors, reads with a Phred quality score  $\geq 20$  were removed, as were sequences below 200 base pairs. Although chimera checking had already been carried out by Research and Testing, additional checks and removals were done with usearch61 using reference based detection against the Silva v119 97% database (Quast *et al.*, 2013).

The new FASTA file generated from the quality filtering described above then underwent microbial diversity analysis. This began with picking OTUs (Operational Taxonomic Units). A number of methods were trialled, including de novo, closed and open reference techniques. Ultimately, we found that open reference OTU picking and taxonomic assignment against the SILVA v119 97% database (Quast et al., 2013) using UCLUST provided good results, with relatively low number of unassigned OTUs. During the OTU

picking process, QIIME was instructed to remove singleton OTUs and OTUs that did not align with PyNAST.

The produced OTU tables were then rarefied to 11582 to remove sampling depth heterogeneity. This involves randomly sampling OTUs amount as common number of sequences between the two files. Rarefaction prevents the sample with more sequences being overrepresented when comparing diversity, since more OTUs will probably be picked from a larger set of sequences, which may incorrectly infer higher diversity when comparing against a sample with fewer sequences. The rarefied OTU table with taxonomic assignment was then used to plot histograms to compare diversity between samples.

## **6.2 Results**

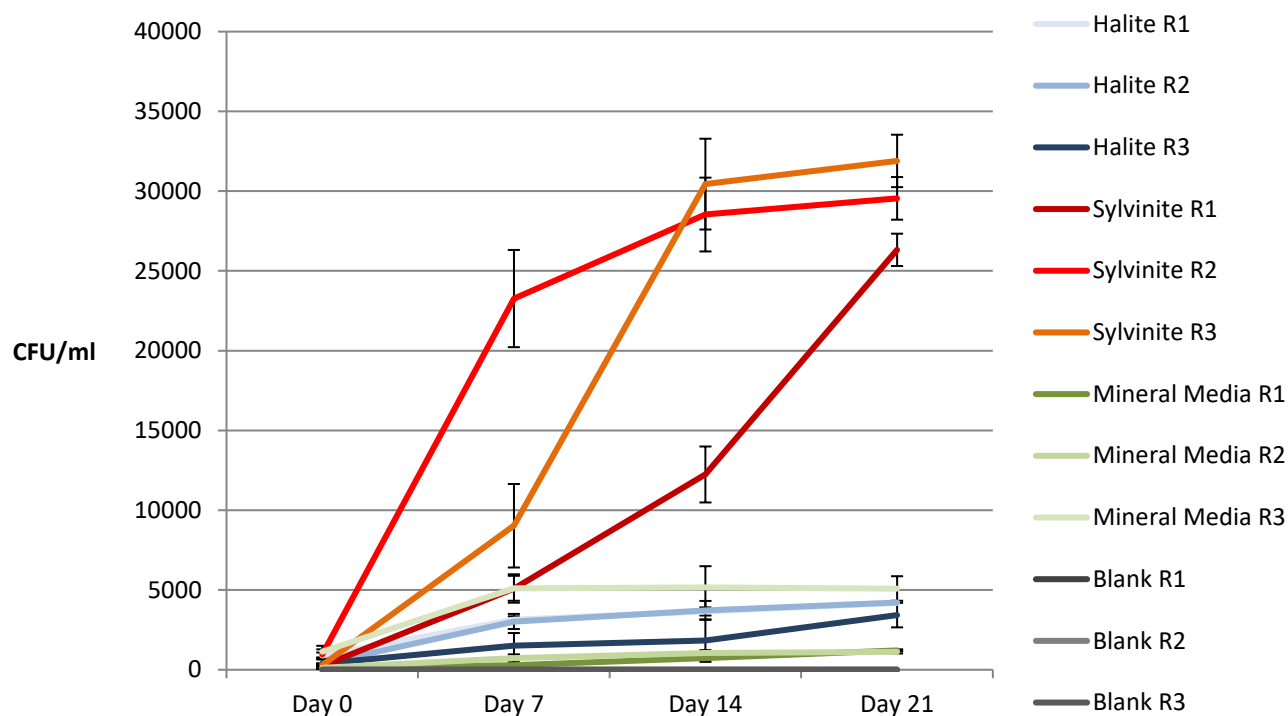
### **6.2.1 Ancient carbon trapped in Permian salt rocks**

The results of experiments 1, A, B and C are detailed individually below.

#### **6.2.1.1 Experiment 1**

Experiment 1 showed that the addition of the halite and sylvinite promoted the growth of the organisms in the brines in comparison to the mineral media (see Figure 6.4). A small amount of growth was detected in the mineral media, despite it not containing any carbon sources. The growth rate and cell density were both much higher in the media containing sylvinite than in halite or mineral medium. The addition of the halite also promoted growth in the communities over the mineral medium. No blanks displayed any growth.

## Experiment 1



**Figure 6.4.** Experiment 1 results displayed as colony forming units per ml in the halite, sylvinite, mineral media and blank media over a period of 21 days. Error bars represent standard error of triplicate colony counts.

The preliminary work in Experiment 1 indicated that the salt rocks could be providing the organisms in the media with carbon. As the highest levels of growth were observed in the sylvinite media, the salt rock containing gaseous hydrocarbons (see Chapter 3), it was hypothesised that the organisms were using carbon sources from within the sylvinite for growth. To better test this hypothesis, a greater number of enrichment cultures were required to control for the geochemical changes brought about by dissolving the salt rocks in the media (see Section 6.1.2.3 for details).

### 6.2.1.2 Experiment A

The results of Experiment A (halite, sylvinite and mineral media inocula) appear to refute the hypothesis derived from Experiment 1. The results have been split into two graphs to reduce

data crowding, these being Figures 6.5 and 6.6. The former shows the sylvinite results and the latter the halite results, with both including the same mineral media and blank data for comparison. Both graphs clearly show the change in ionic composition was the key influence on growth as exhibited by the comparable growth rates/cell densities in the controls compared to the natural unmodified salt rocks.

Cell densities were again significantly higher in the sylvinite media and all its controls. The fastest growth rates were found in the artificial sylvinite media. Communities in furnace sylvinite and sylvinite media both grew a little slower. However, the final CFUs/ml values for the controls and sylvinite media were within the errors of one another. The only exception being one of the artificial sylvinite replicates, which after 54 days displayed ~6000 CFUs/ml above the other sylvinite and halite media replicates and their respective controls.

The halite media also showed similar patterns. It consistently displayed higher growth rates than in the mineral media as did its controls. Two of the triplicate halite media had CFU/ml values of around 5000 after 54 days. The remaining halite media replicates and the rest of the controls all had similar CFU/ml counts throughout the 54-day experiment.

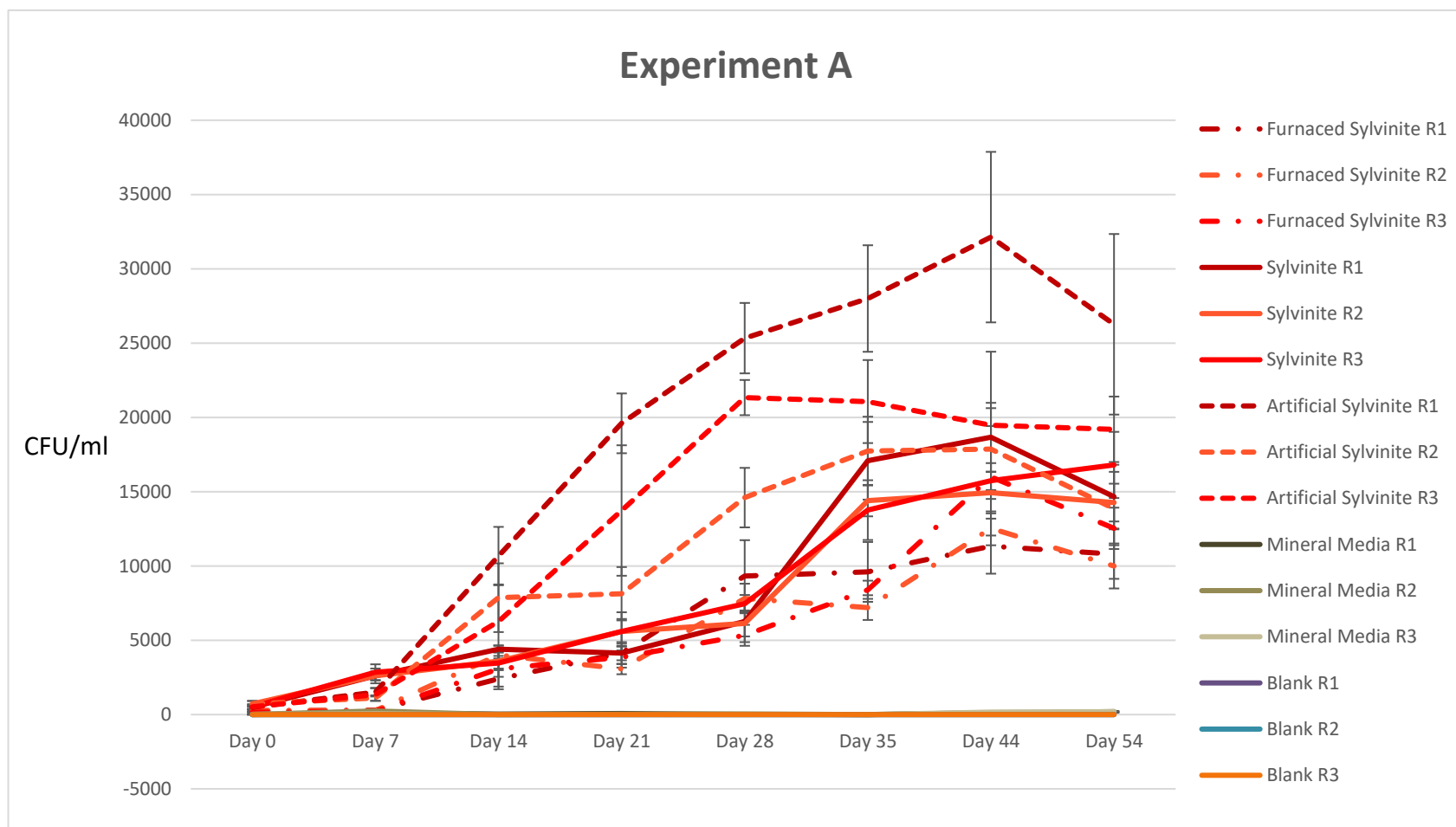
### **6.2.1.3 Experiments B and C**

CFUs/ml counts were generally an order of magnitude lower in Experiments B and C, which were inoculated exclusively with the mineral media. Experiment C used a similar set-up to Experiment B, with the only difference being the lack of media autoclaving and the filtering of the insoluble materials in the salt. The data for each experiment is also split up into two graphs for the same reason as Experiment A. Both show similar results to Experiment A in regards to the change in ionic composition being the key influence on growth.

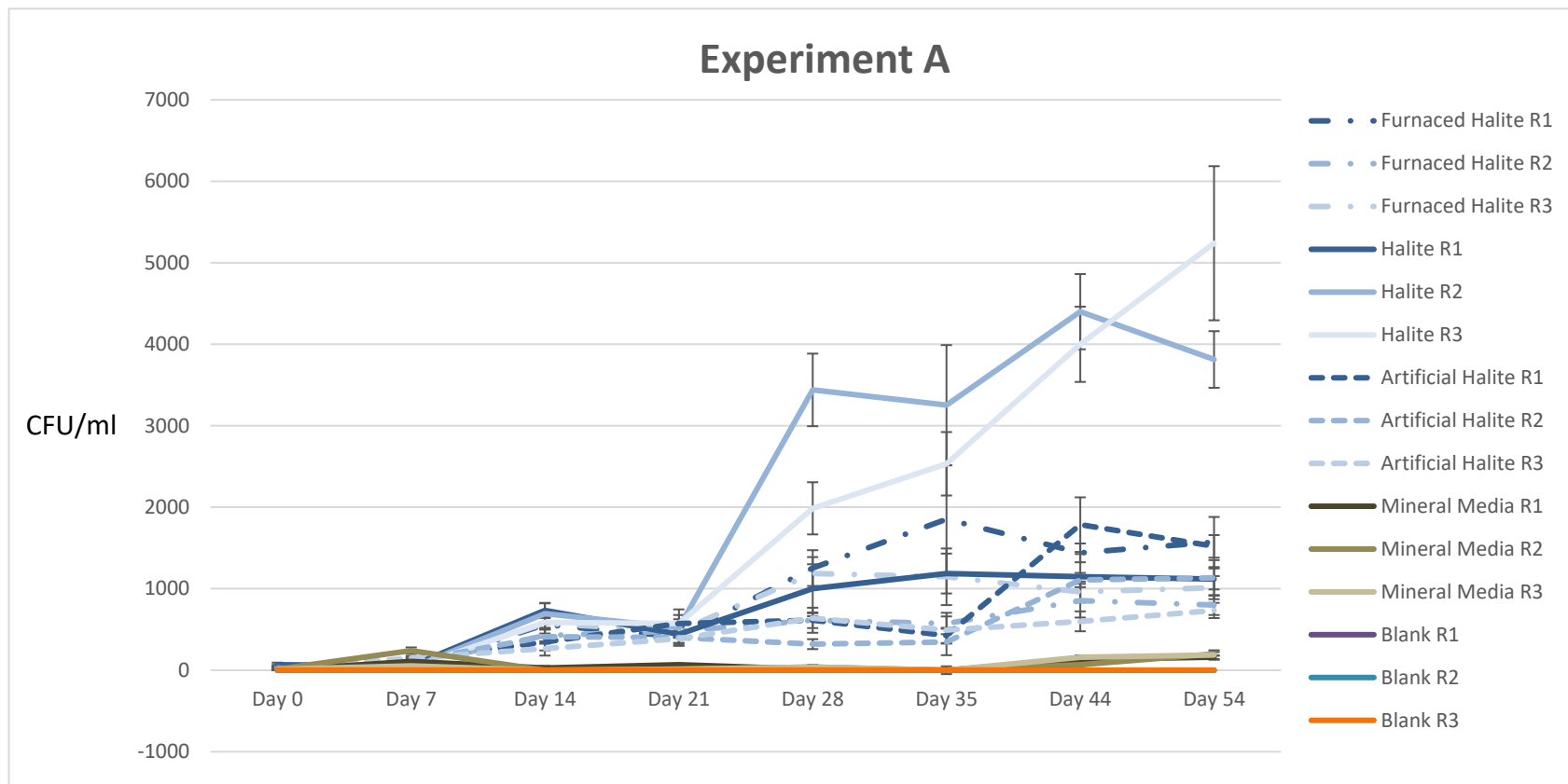
In Experiment B (Figures 6.7 and 6.8), the growth curves display a more rapid increase in CFU's between the days 28 to 35. The majority of the cultures then begin a process of decline. Any stationary phase between day 28 and 44 is short lived and only captured by one time point due to the sampling resolution. The last CFU/ml averages were highest in the sylvinite media, but generally cell counts were similar to the furnace sylvinite control. Cell counts were lower in the artificial sylvinite media consistently, post lag phase. Unlike in Figure 6.5 and 6.6 (Experiment A), growth in the halite media and controls is generally comparable to that in the sylvinite media. The one exception is the artificial halite media, which displayed similar growth levels to that found in the mineral media.

Cell counts were generally lower in Experiment C (Figures 6.9 and 6.10) than in the autoclaved media in Experiment B. Again, cell counts were highest in the sylvinite media, followed by its controls. Growth in the halite media and its controls are again similar that observed in the sylvinite.

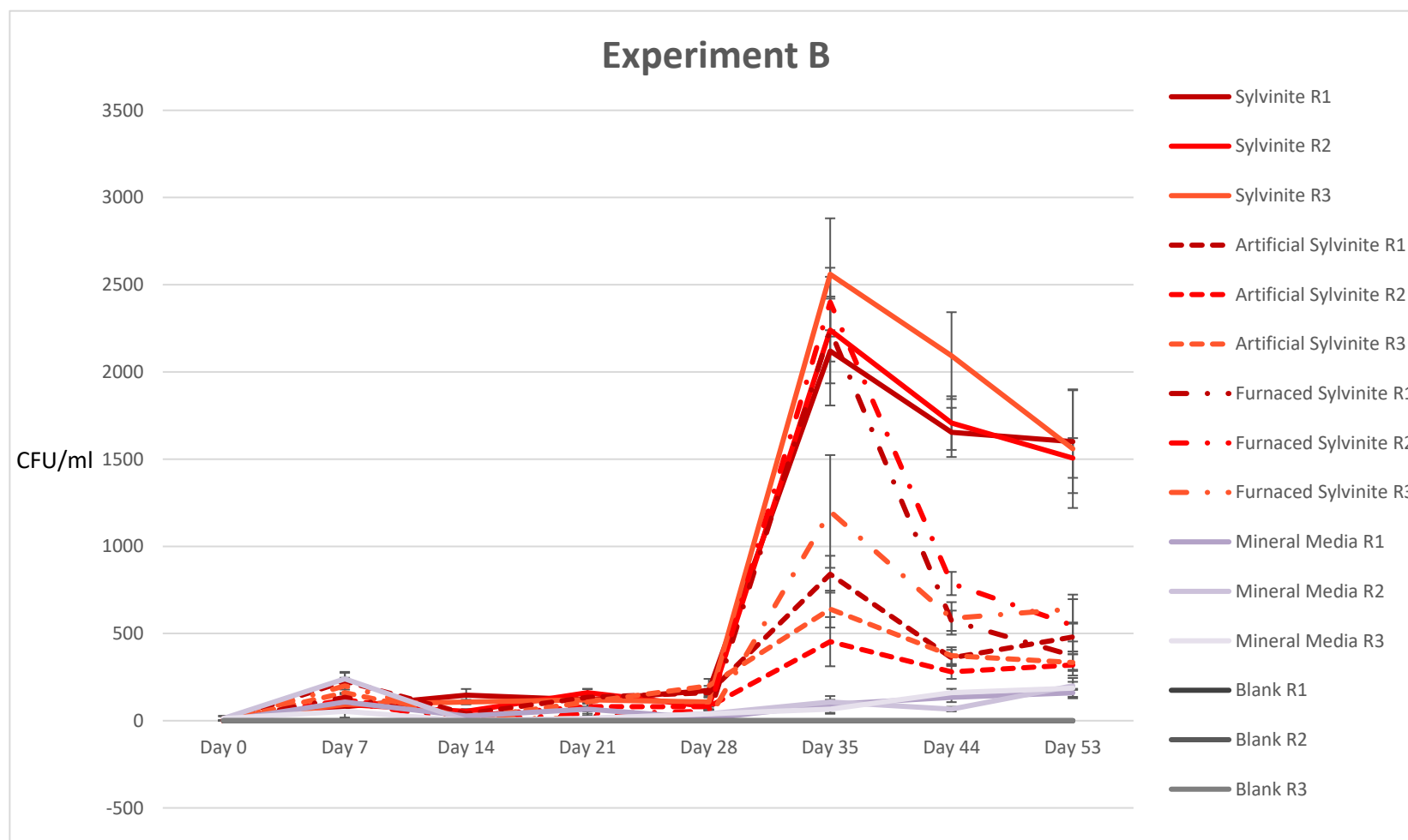




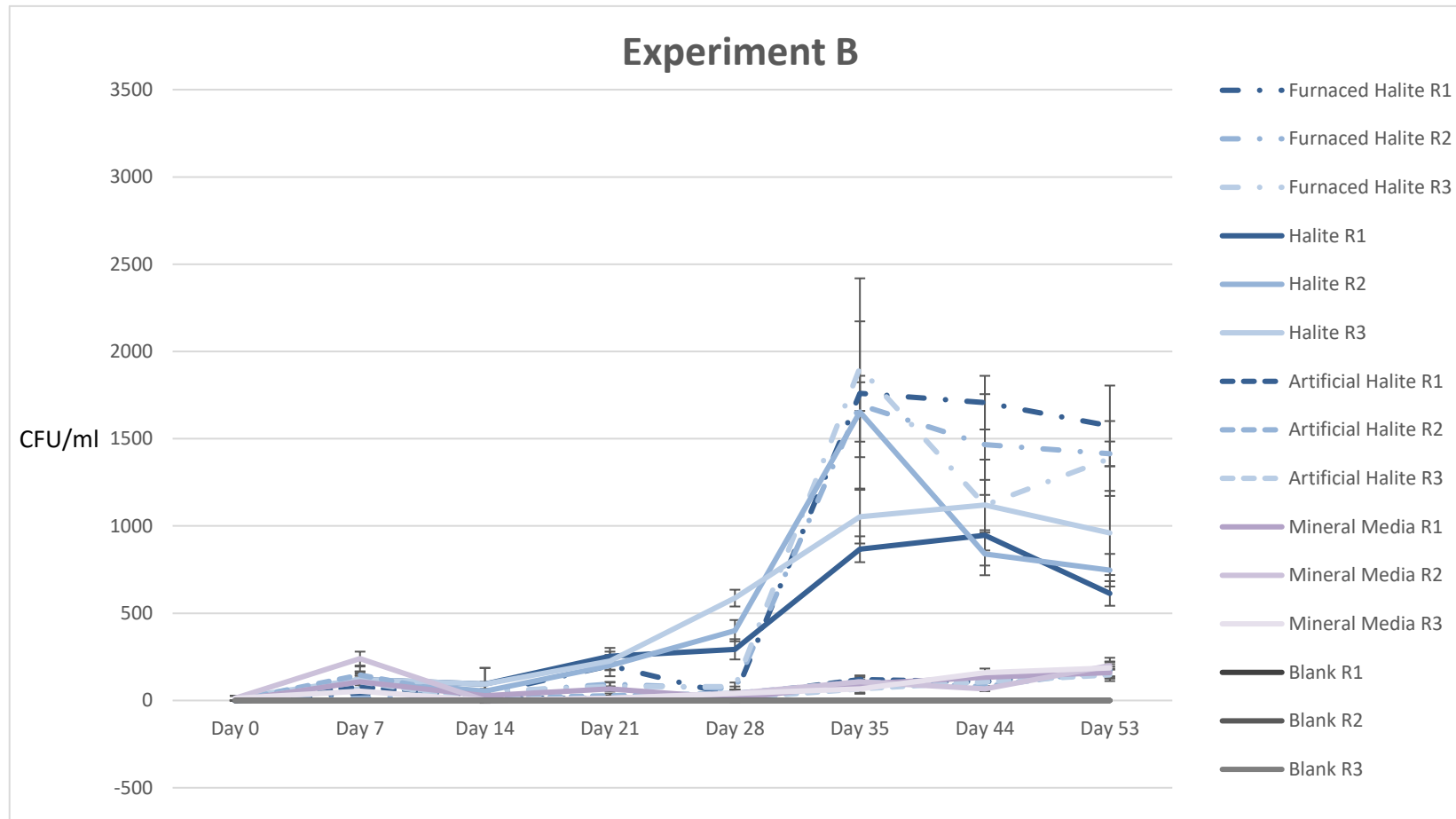
**Figure 6.5.** Growth rates of cultures in sylvinite and mineral media over 54 days. Three different inoculum types were used for the experiment (see Figure 6.3). The controls for the sylvinite salt rock media display similar growth patterns, suggesting that the change in ionic composition of the media is largely responsible for the change in growth observed. Error bars represent standard error of triplicate colony counts.



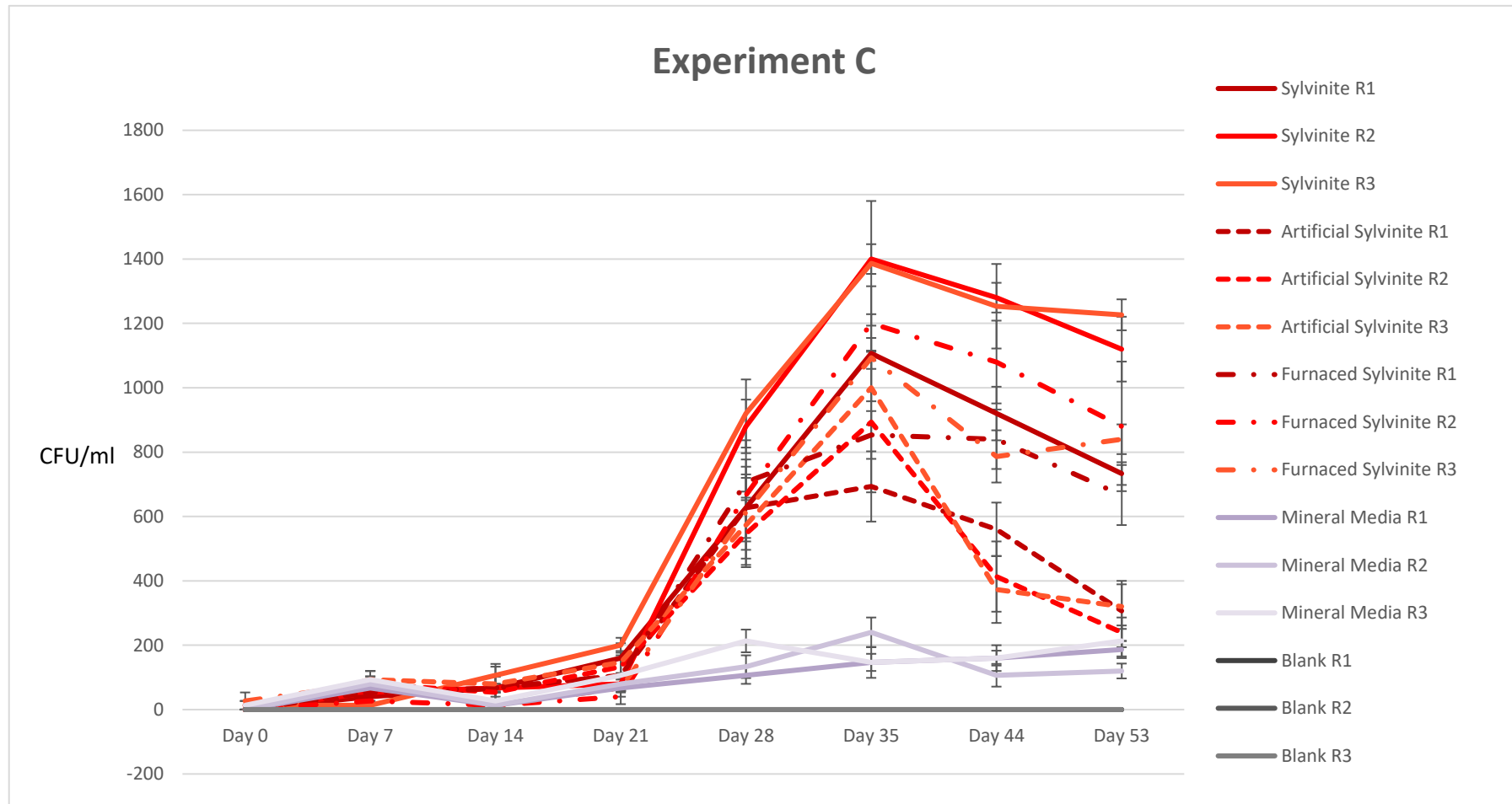
**Figure 6.6.** Growth rates of cultures in halite and mineral media over 54 days. Three different inoculum types were used for the experiment (see Figure 6.3). Note, the scale is different to Figure 6.5 (retaining the same scale made the data difficult to read). The mineral media and blank data is identical to 6.5. The halite produced less growth than the sylvinite media, but was still above the mineral media. There was a separation between two of the unaltered natural halite media replicates and the other controls. However, the third replicate showed less growth than the controls. Error bars represent standard error of triplicate colony counts.



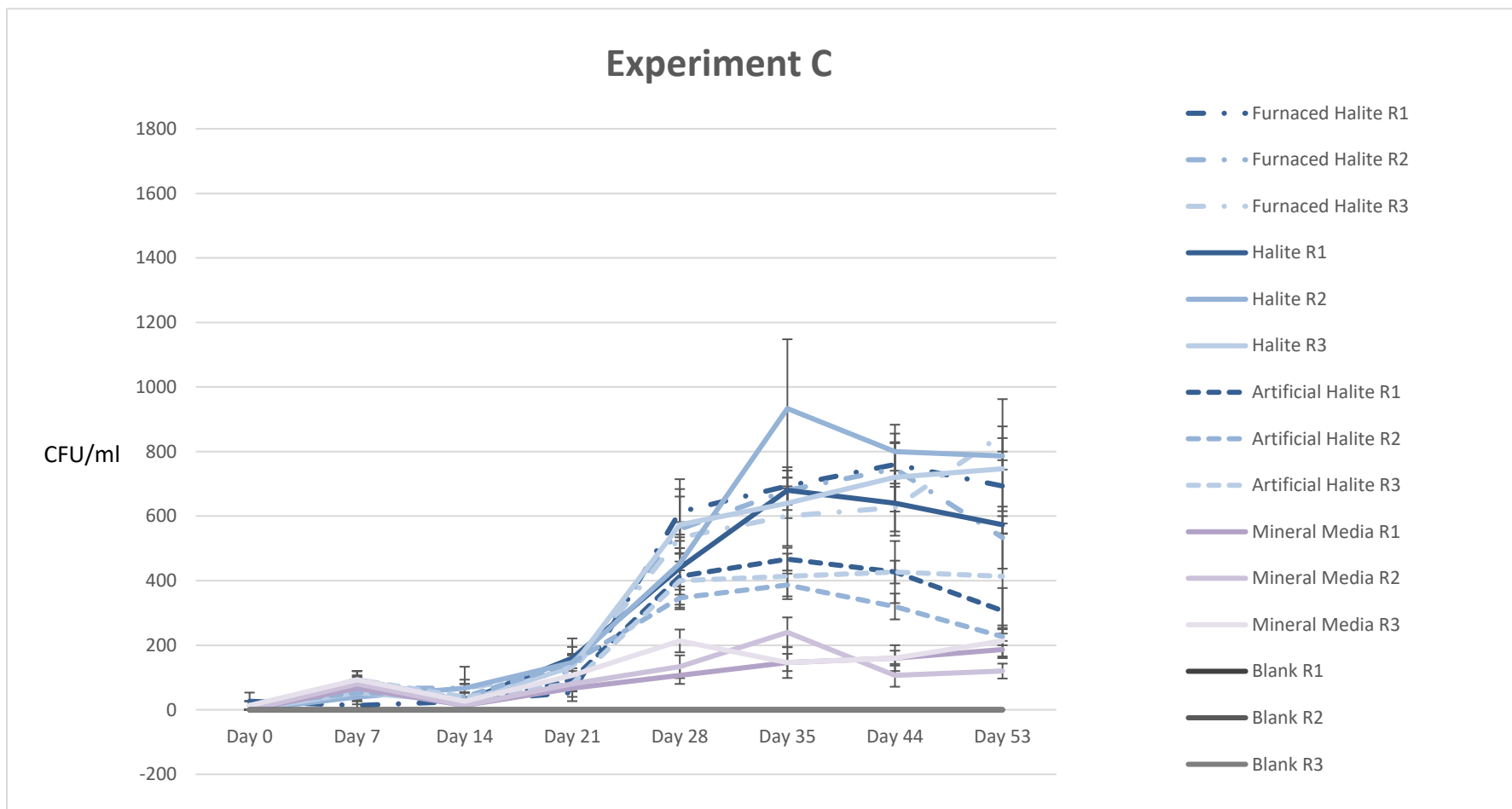
**Figure 6.7.** Growth rates of cultures in sylvinite and mineral media over 53 days. This time a single inoculum type for all the media variants was used (see Figure 6.3). Again, little difference in growth exists between the controls and the test media suggesting geochemistry was again the overall controlling factor in determining growth. Error bars represent standard error of triplicate colony counts.



**Figure 6.8.** Growth rates of cultures in halite and mineral media over 53 days. A single inoculum type was used for all the media variants (see Figure 6.3). Little difference in growth exists between the controls and the test media suggesting geochemistry was again the overall controlling factor in determining growth. The mineral media and blank data is identical to 6.7. This experiment showed less separation in growth between the halite and sylvinite media. Error bars represent standard error of triplicate colony counts.



**Figure 6.9.** Growth rates of cultures in sylvinite and mineral media over 53 days. As in Experiment B, the same single inoculum source was again used for all the media types. However, the media was not autoclaved, but sterilised by filtration. The results are similar to Figure 6.7, suggesting the two method of sterilisation made little difference to the outcome of the experiment, although the overall CFUs/ml are lower. Error bars represent standard error of triplicate colony counts.



**Figure 6.10.** Growth rates of cultures in sylvinite and mineral media over 53 days. As in Experiment B, the same single inoculum source was again used for all the media types. However, the media was not autoclaved, but sterilised by filtration. The results are similar to Figure 6.8, with the overall CFUs/ml again being lower. The mineral media and blank data is identical to 6.9. Error bars represent standard error of triplicate colony counts.

## 6.2.2 Community carbon utilisation capabilities

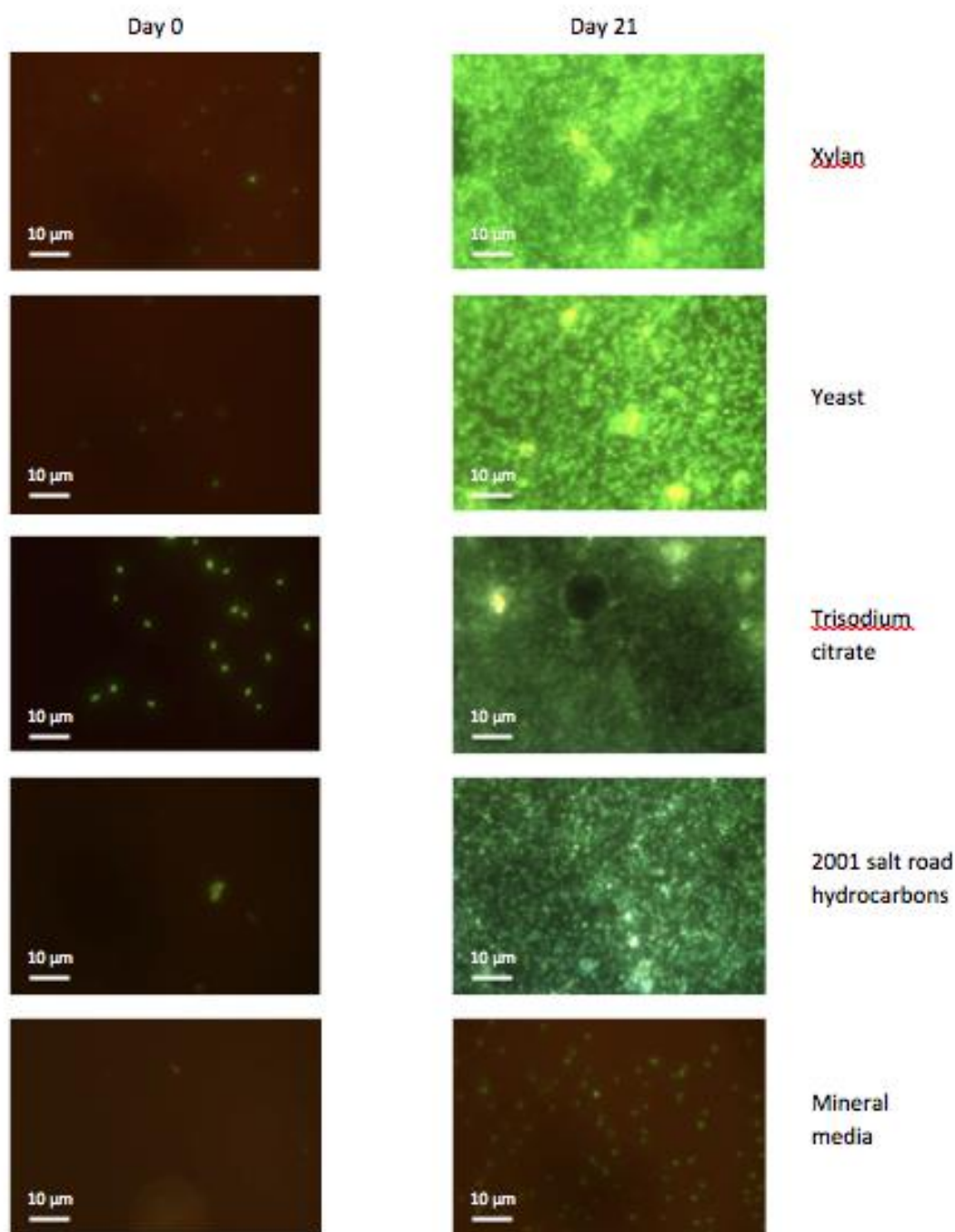
The media described in Section 6.1.3 were able to produce a number of positive enrichments. The results are displayed in Table 6.5.

		+/- growth for each triplicate	
Inoculant	Carbon Source	Aerobic	Anaerobic
Billingham baths	Yeast extract	+ / + / +	+ / + / +
215	Yeast extract	+ / + / +	+ / + / +
Billingham baths	Glucose	+ / + / +	- / - / -
215	Glucose	+ / + / +	+ / - / +
Billingham baths	Xylan	+ / + / +	+ / + / +
215	Xylan	+ / + / +	+ / + / -
Billingham baths	Trisodium citrate	+ / + / +	+ / + / +
215	Trisodium citrate	+ / + / +	+ / + / +
Billingham baths	2001 salt road hydrocarbons	+ / + / +	+ / + / +
215	2001 salt road hydrocarbons	+ / + / +	- / - / -
Billingham baths	L1026 hydrocarbons	+ / + / +	- / - / -
215	L1026 hydrocarbons	- / - / -	- / - / -

**Table 6.5.** Aerobic carbon usage in enrichments inoculated with deep subsurface brines.

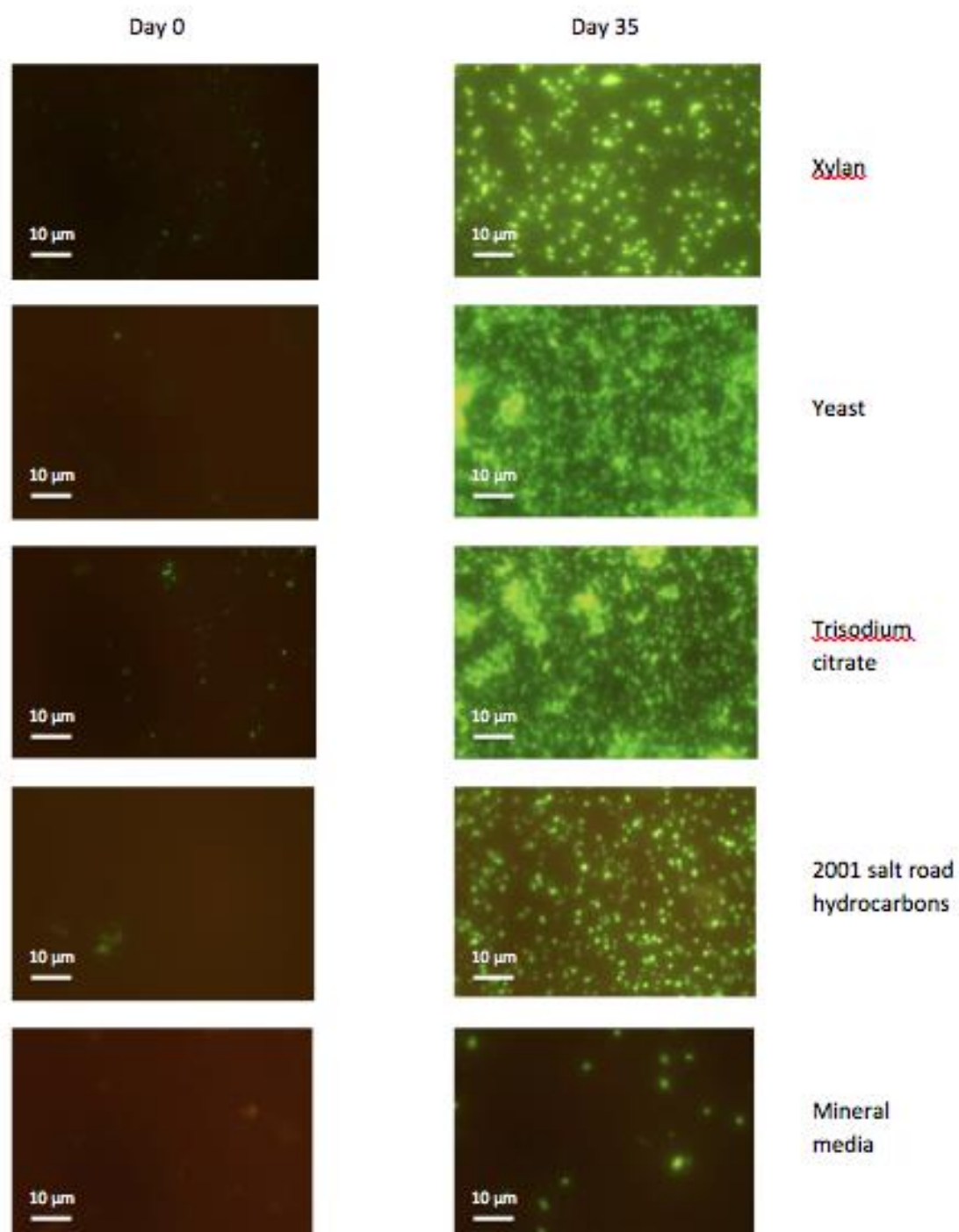
Growth was observed on all the carbon sources tested in aerobic conditions. Growth was not observed in any cultures in the anaerobic conditions using the L1026 hydrocarbons as a carbon source. It is worth noting that low levels of growth were again observed in the mineral media. Boxes marked with a + displayed growth levels above that observed in the mineral media.

The community was able to utilise a range of carbon sources and grew successfully on all the aerobic carbon sources tested, including the two oil seeps. Anaerobically, enrichment was less consistent, with growth being observed on the 2001 salt road hydrocarbons when inoculated from Billingham Baths, but not 215. Growth was not detected when using either brine to inoculate anaerobic media containing the L1026 hydrocarbons. Microscopy of some of these cultures is displayed on Figures 6.11 and 6.12.



**Figure 6.11.** Microscopy of a number of aerobic cultures after 21 days inoculated with Billingham Baths. The stain used was SYBR Gold.



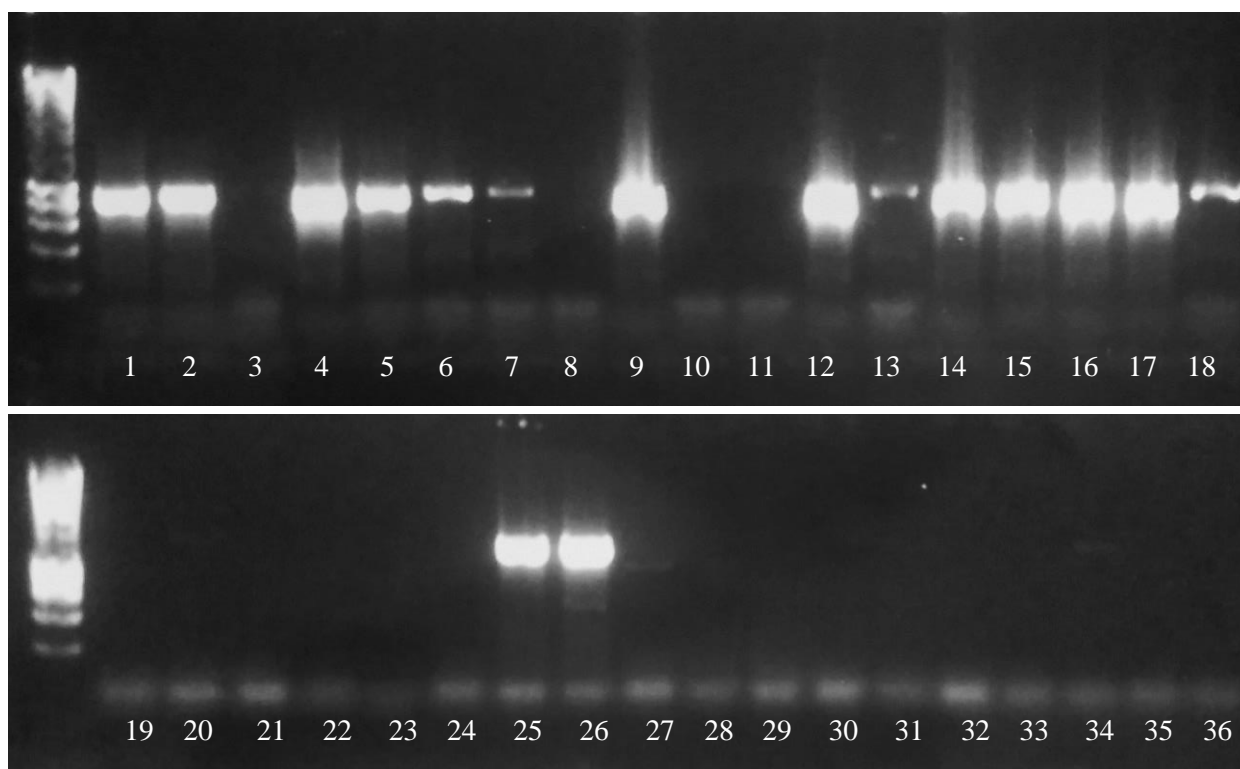


**Figure 6.12.** Microscopy of a number of anaerobic cultures after 35 days inoculated with Billingham Baths. The stain used was SYBR Gold.

### 6.2.3 DNA and sequencing

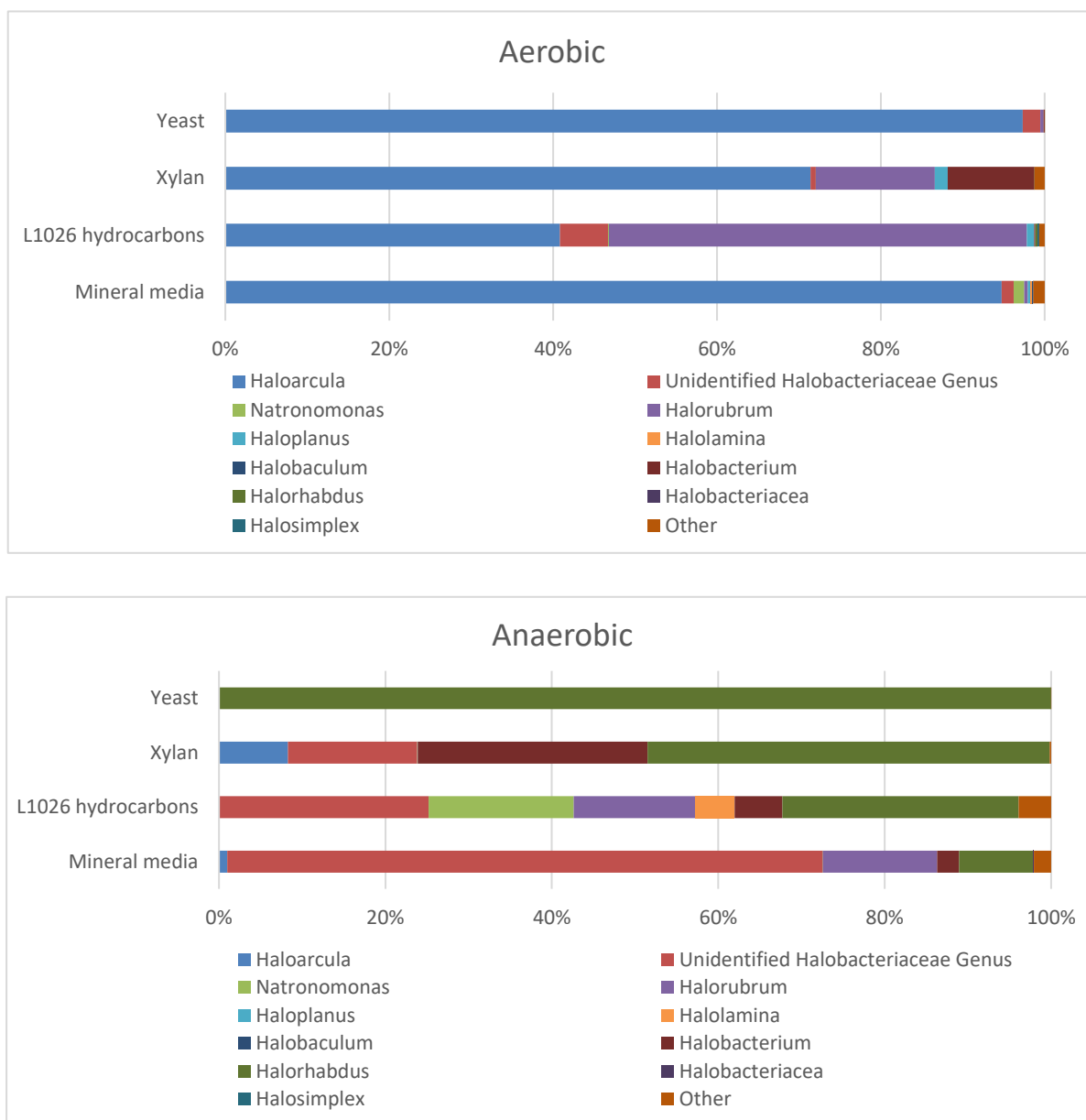
A subset of the enrichment cultures listed above were amplicon sequenced. The yeast extract culture was selected to examine growth with a complex carbon source, xylan, a more recalcitrant long chain sugar and the mineral media to determine the organisms capable of growing in an oligotrophic environment. The light hydrocarbons were also selected to examine which parts of the brine microbial community would be able to utilise the hydrocarbon seeps best. Additionally, these carbon sources all supported growth under anaerobic conditions, allowing a comparison to be made between the two environments.

The cultures had been transferred three times before sequencing and then been allowed to grow to a stationary phase. PCR indicated that archaea dominated the enrichment cultures (Figure 6.13) meaning only archaeal primers were used for amplicon sequencing.



**Figure 6.13.** PCR products post gel electrophoresis imaged under UV from carbon enrichment cultures. Archaeal (21F/958R) (samples 1-18) and bacterial (27F/1389R) (samples 19-36) primers were used for PCR. Some non-specific binding occurred, seen at the base of each gel lane. Some very faint products were present in the PCR using bacterial primers in the yeast and xylan, but generally the archaea clearly dominate the cultures. Key: 1/19, 2001 salt road hydrocarbons. 2/20, xylan. 3/21, blank media. 4/36, yeast extract. 5/22, trisodium citrate. 6/23, glucose. 7/24, mineral media. 8/31, negative PCR control (dH<sub>2</sub>O). 9/28, L1026 hydrocarbons. 10/30, anaerobic L1026 hydrocarbons. 11/29, anaerobic glucose. 12/27, anaerobic yeast extract. 13/32, anaerobic mineral media. 14/33, anaerobic 2001 salt road hydrocarbons. 15/34, anaerobic xylan. 16/25, positive control (*Haloferax volcanii* DNA/*E.coli* DNA). 17/26, mineral media spiked with positive control and then DNA extracted. 35/18, anaerobic trisodium citrate.

The archaeal DNA was then sequenced on the Illumina MiSeq platform. The histograms below display the community composition assigned through using the Qiime pipeline described in Section 6.1.4.



**Figure 6.14.** Histograms displaying the composition of the archaeal genera identified by 16S pyrosequencing in hypersaline enrichment media containing several different carbon sources. *Haloarcula*, and to a lesser extent *Halorubrum*, dominate the aerobic cultures. The most abundant genera switch in the anaerobic media to an unidentified genus within *Halobacteriaceae* and *Halorhabdus*.

*Haloarcula* generally dominate the aerobic cultures, particularly in the yeast and mineral media where it makes up >95 % of the community. Both xylan and the 2001 salt road hydrocarbons had slightly small proportions of *Haloarcula*, at 70 % and 41 % respectively.

*Halorubrum* appeared in significant quantities particularly in the 2001 salt road hydrocarbons making up 51 % of the hits, and xylan at 15 %. Small numbers of hits were also attributed to a variety of archaeal genera, including *Natromonous*, *Haloplanus* and *Halosimplex*.

The archaeal community markedly shifts when examining the anaerobic cultures. Small portions of *Haloarcula* do still appear, particularly in the xylan where it makes up 8 % of the hits. The genus *Halorhabdus* is far more common in the anaerobic cultures, making nearly the entirety of the yeast media at 99.9 % of the hits. It is also a significant part of the xylan, 2001 salt road hydrocarbon and the mineral media, making up 48 %, 28 % and 9 % of the hits. The other significant genus in the anaerobic cultures is an unidentified genus within *Halobacteriaceae*. This makes up nearly 72 % of the hits in the mineral media, and was also identified in the 2001 salt road hydrocarbon and xylan media at 25 % and 16 % respectively. Generally, the 2001 salt road hydrocarbon media is more evenly split into different genera rather than being dominated by one genus. *Halorubrum* and *Halobacterium* also make appearances in the 2001 salt road hydrocarbon media at 15 % and 6 %, and in the mineral media at 13 % and 9 %.

## 6.3 Discussion

### 6.3.1 The impact of Permian salt rocks on microbial propagation

The preliminary experiment indicted a clear distinction between the numbers of colony forming units in the halite and sylvinite media. Given the lack of a carbon source in the media and the sylvinite containing gaseous hydrocarbons (see Chapter 3), it was initially hypothesised that this additional growth was related to carbon found within the sylvinite. Upon adding the furnaceed and the artificial sylvinite controls, it was clear this hypothesis was incorrect. Growth was also greatly promoted above the mineral media in both additional controls, indicating that the shift in aqueous ionic composition was responsible for the additional growth, not the carbon present within the sylvinite. The same reasoning is applicable to the results from the halite media, which again demonstrated that the change in aqueous ionic environment was responsible for the extra growth.

Whilst these results suggest that any carbon present in the salt rocks did not impact growth in the experiments, the addition of the salt rocks did promote growth through changing the geochemical make-up of the media. As discussed in Chapter 2, extreme halophiles require  $K^+$  as an intercellular osmoprotectant (e.g. Ortega et al. 2011). By introducing sylvinite into the media, the concentration of  $K^+$  ions was greatly increased and probably responsible for the fertilisation effect observed. ICP-OES and IEC both showed that the halite also introduced a smaller, but still significant component of K into the media. This is most likely related to the minor sylvite component within the halite (see Section 3.3.2.1) and may have been the reason for the more modest growth increases observed in this condition. The addition of a number of other ions and trace elements could also have had an impact on growth. Whilst these results do not suggest that any carbon present in the salt rocks was a factor in promoting growth, it does indicate that cell numbers may increase in certain regions of the deep subsurface evaporite deposit as brines contact different horizons if a usable carbon source is present.

Overall CFUs/ml varied between Experiments A, B and C. The two experiments (B and C) inoculated with the mineral media both displayed approximately an order of magnitude less CFUs/ml in comparison to the halite and sylvinite media in Experiment A (which were inoculated with the sylvinite and halite media). This could be related to several factors, including the different media types selecting for organisms that grow to different cell densities and that were better adapted to the media conditions. Alternatively, the inoculum from the halite and sylvinite media delivered more organic matter to the test media than the mineral media. A further decrease in cell density was also observed between the two experiments (B and C) using the mineral media as an inoculum. This may have been related to the lack of insoluble material in Experiment C (lost due to filter sterilisation), which could have provided additional surfaces for the organisms to grow on. There is no evidence from Experiments A and B, that this insoluble material contained usable carbon sources.

These experiments found no evidence that halophilic communities in the Zechstein can utilise any trapped carbon found within the salt rocks. Further work (see Section 6.4) using different set-ups and sampling different salt rocks, could yet show that some of the salt rocks do contain usable carbon sources. Other potential carbon sources in the Zechstein are discussed below.

### 6.3.2 Carbon usage in the deep subsurface community

Despite no evidence being found to suggest that the halophiles present in Boulby can utilise carbon within the ancient salt rocks, it does appear that the growth of these organism is greatly increased when they encounter oil seeps from the mine. They were also able to utilise a range of carbon sources under aerobic conditions where growth was successful in all conditions except the L1026 hydrocarbon culture inoculated with brine 215.

In anaerobic conditions this carbon usage seems to be more restricted, with enrichment on the L1026 oil seep failing to produce additional growth compared to the control. Without carrying out a detailed investigation into the exact changes occurring in the seeps during enrichment, it is not possible to know exactly which portion of them is being used by the halophiles. However, it is possible to hypothesise that the lack of growth in the anaerobic L1026 hydrocarbons, despite growth in the 2001 salt road oil seep, is most likely related to the difference in composition between the two hydrocarbons. As shown in Table 6.4, L1026 hydrocarbons are much higher in aromatic molecules as opposed to alkanes. They also contain more polyaromatic hydrocarbons and roughly the same asphaltenes. The higher portions of aromatic and polyaromatic hydrocarbons in L1026 may be responsible for these different growth responses. Generally, alkanes and lower molecular weight hydrocarbons are the most susceptible to biodegradation, and aromatic and polyaromatic hydrocarbons are less susceptible and often toxic. The asphaltenes are very resistant to biodegradation (see Wrenn and Venosa 1996). Higher proportions of aromatic and polyaromatic hydrocarbons and lower quantities of alkanes present in L1026, makes it more difficult to utilise and potentially toxic. The hydrocarbon to none hydrocarbon (HC/non-HC) ratio is also lower in L1026, meaning that not only are the hydrocarbons generally longer chain and more difficult to metabolise, less of them are present per volume added. These factors, combined with the anaerobic environment and high concentrations of NaCl, seems to have been responsible for the lack of growth in the enrichments using L1026 as a carbon source.

The organisms growing on these carbon sources changed with the different enrichment conditions. Whilst organisms in the Halobacteriaceae family dominate the aerobic and anaerobic cultures, the genera present changed. *Haloarcula*, a commonly enriched halophile in hypersaline aerobic cultures, dominated the aerobic cultures showing the metabolic flexibility of the genus, but became a more minor component in the anaerobic cultures. Instead the *Halorhabdus* genus, which contains species capable of living in anaerobic or

suboxic conditions (e.g. Antunes et al. 2008b), dominated along with an unidentified genus of Halobacteriaceae.

Both *Haloarcula* and *Halorubrum* were common in the aerobic 2001 salt road oil seep media. Species within these two genera have been shown to be able to degrade hydrocarbons under aerobic conditions (e.g. Tapilatu et al. 2010, Erdoğan et al. 2013) and may be carrying out the same process here. A greater variety of genera were found in this media under anaerobic conditions, making it difficult to hypothesise which, if not all, genera detected in the sequencing could be potentially utilising the hydrocarbons. The lack of characterised anaerobic hydrocarbon degrading halophiles in the literature also makes this difficult (Martins and Peixoto 2012, Fathepure et al. 2015). The genera *Halorubrum* and *Halobacterium*, both detected in the media, do contain characterised strains able to utilise hydrocarbons (Al-Mailem et al. 2010, Erdoğan et al. 2013) and live under anaerobic conditions (Tomlinson et al. 1986, Kondo et al. 2015), but both abilities have not been demonstrated together.

Despite genera containing organisms able to use hydrocarbons being detected in the cultures, the growth could still be related to the non-hydrocarbon fraction of the oil seeps. This may be supported by the metagenomics work from Chapter 5. A number of the same genera containing species known to be hydrocarbon degraders were also found in the metagenomes where hydrocarbon degradation pathways were absent, demonstrating their presence is no guarantee of biological hydrocarbon degradation. Alternatively, the absence of pathways related to hydrocarbon degradation in the metagenomes may simply be caused by genes related to these pathways being present in very low concentrations and were therefore not captured by the sequencing. Additionally, pathways related to these metabolic processes are not well characterised, especially in relation to hydrocarbon degradation in halophiles (Fathepure 2015), meaning poorly understood hydrocarbon degradation pathways may have been present in the metagenome.

Although this enrichment work does not definitely prove that some halophiles in the mine can utilise the hydrocarbon component of the oil seeps as a carbon source, it does show that these seeps promote the growth of halophiles in anaerobic environments and presumably provide some form of carbon source (even if it is not the hydrocarbons). Oil seeps could therefore be an important carbon source over geological time for halophiles in the Zechstein sequence, even when oxygen is unavailable.



Despite no carbon source being added to the control mineral media, very low levels of growth were consistently observed in these controls. Halophiles have been shown to possess an ability to grow in oligotrophic environments using a number of strategies, such as changes in cell morphology and polyploidy (e.g. Bolhuis et al. 2005, Zerulla et al. 2014, Stan-Lotter and Fendrihan 2015). These adaptations are probably responsible for this ability to grow in the absence of a significant carbon source. The high quantities of salts added to the media would have likely carried trace amounts of carbon into the media, contributing to this low level of growth.

The abilities of the brine communities to grow in the mineral media and utilise a range of carbon sources demonstrates their suitability to grow in environments with slow environmental turnover. Based on their TOC content (Chapter 3.4.4), the brines probably contain adequate amounts of carbon to support microbial activity for a period provided these carbon sources are in a form that can be utilised by the microbes. As discussed below, examining the types of carbon present in the brines and then testing brine community growth on them is a natural next step for this work.

## **6.4 Limitations and further work**

The experimental design utilised here necessitated a number of limitations that are worth outlining so they can be addressed in further work. In the salt rock enrichment experiments, the growth periods could be extended. Whilst the community had entered the decline phase according to the colony counts by the end of the time points, it may take very long periods for these organisms to breakdown any extremely recalcitrant carbon sources present. Other methods of quantifying growth could also be attempted since the agar plates are inherently biased towards organisms able to form colonies on plate surfaces. Further work could also expand to include a greater sampling range of salt rocks and brine inoculum from around the mine.

Whilst it was suspected that the detrital material present within the salt minerals probably contained carbon compounds due to previous work (Griffith et al. 2008), the carbon compounds present in the salt minerals collected, in addition to the gaseous hydrocarbons examined in Chapter 3, were not determined. Samples of the salt rocks were provided to

collaborators to examine their carbon contents, but this proved technically challenging and the work is on-going. Once these results are available, it may be prudent to attempt enrichment experiments on these carbon sources in isolation. Whilst this may be more artificial than enriching communities on media amended with natural unaltered salt minerals, it may be more effective at highlighting usable carbon sources. Once the carbon sources present are determined, constraining the enrichment time periods required to test for growth might also be more easily estimated. Additionally, enrichments on these salt rocks were not carried out under anaerobic or microaerophilic conditions which better mimic the deep subsurface environment. Although it is unlikely that the halophiles present in the brines would be more capable at accessing carbon sources under anaerobic conditions.

Whilst basic TIC/TOC was carried out on several brines, no detailed examination of the exact organic compounds present was undertaken. Testing community growth on these organic compounds could isolate additional usable carbon sources. Moreover, this work could identify the origin of the organic carbon in the brines, providing an improved understanding of brine origin and evolution and carbon sources and sinks in the deep subsurface.

Work on the oil seep enrichments could also be expanded in several ways. As outlined, the growth experiments on the two oil seeps were designed to examine if the addition of these oils would increase growth in the halophilic communities. However, they did not include any examination of the hydrocarbons during this growth period, making this the natural next step for this work. This would determine why the addition of these seeps promoted growth and whether, as suspected, this was related to the usage of any hydrocarbons. Additionally, enrichments could be carried out on a range of hydrocarbons detected in the oil. This would simplify the work involved in monitoring the hydrocarbons present and any degradation occurring.

## **6.5 Conclusion**

This chapter aimed to examine several ways halophiles in deep subsurface evaporite deposits obtained carbon for growth using enrichment techniques. From this work a number of conclusions can be drawn:

- Within the confines of the experimental design, no evidence was found that any carbon entrapped within the salt rocks could provide the organisms found in the brines with a usable carbon source. All changes in growth could be attributed to the geochemical changes brought about by the shift in media ionic composition when dissolving the salt rocks.
- Despite this negative result, a fertilisation effect was observed when the salt rocks were added to the media. This indicates that brine interactions with certain evaporite layers containing higher concentration of mineral like KCl, may result in more favourable conditions for growth in these deep subsurface brines. As demonstrated by the growth consistently observed in the mineral media, only low levels of organic carbon would be required for this growth to occur.
- Deep subsurface halophilic communities can grow in oligotrophic environments as demonstrated by their propagation in the mineral media. This ability has been observed by other authors examining isolated halophiles (Bolhuis et al. 2005, Zerulla et al. 2014) and appears to be retained by deep subsurface brine communities. With the potential scarcity of organic carbon in some deep subsurface environments (Purkamo et al. 2015) this would seem an important adaptation.
- Oil seeps entering the Zechstein sequence promote growth of the deep subsurface communities. The communities seem able to grow on a greater range of oil seeps under aerobic conditions, but could still utilise the 2001 salt road oil seep under anaerobic conditions. Whilst further work would be required to determine which carbon sources found in the oil seeps were being used by the organisms, sequencing found a number of genera in the aerobic and anaerobic cultures that contain organisms shown to degrade hydrocarbons.
- Generally, the deep subsurface community in Boulby is able to utilise a range of organic compounds for growth under both anaerobic and aerobic conditions, although that range appears more restricted under anaerobic growth. Metabolic flexibility would be an important asset to organisms trying to survive in deep subsurface evaporites.

These data demonstrate that deep subsurface halophile communities in Boulby mine and likely the wider Zechstein sequence are well adapted to slow environmental turn over and can take advantage of a range of carbon sources when they become available. Most likely carbon sources transported in through the movement of waters and oils in the deep subsurface, as demonstrated in chapter, all contribute to providing these communities with small amounts of carbon over geological time.

## **Chapter 7: Dissimilatory sulphate and iron reduction in a deep subsurface evaporite deposit**

The large quantities of sulphate and ferric iron minerals on Mars (e.g. Christensen et al. 2001a, b, Gendrin et al. 2005) and the lack of oxygen in its atmosphere have lead a number of authors to suggest that dissimilatory sulphate and Fe(III) reduction are two metabolisms that could be used by extant Martian life (e.g. Marnocha et al. 2010, Nixon et al. 2013, Parnell et al. 2016). The inhospitable surface conditions present on the planet makes its deep subsurface one of the best places to search for life.

As discussed in Chapter 2, evaporite deposits are a common feature on the surface of Mars and likely extend into the deep subsurface. Little is known about iron metabolism in hypersaline environments (Emmerich et al. 2012), particularly in the deep subsurface. Some studies have been carried out on sulphate reducers in deep subsurface hypersaline environments, but these are limited to a small number (e.g. Avrahamov et al. 2014). This work will help understand whether these Mars relevant metabolisms are present in deep subsurface evaporite brines, and help expand the known biogeography of these metabolisms, which particularly in the case of microbial iron reduction, is limited.

This shorter chapter tests for the presence of these two metabolisms in the deep subsurface brines using enrichment techniques. Unfortunately, due to an incubator malfunction, the enrichment cultures were destroyed before they could be sequenced. The long growth times meant it was not possible to recover them within the time frame of the project, so this aspect of the work could not be completed. Whilst omitting this work altogether from this thesis was considered, it was decided that the results were of potential interest to future workers and were therefore worth including.

## 7.1 Methods

All sampling methods used were identical to those described in Section 4.1.1.

### 7.1.1 Enrichment of iron reducers

Enrichments were set up for iron reducers using the same anaerobic set-up and techniques described in Chapter 4 and are similar to those described in Nixon and Cockell (2015). Before the base media was created, ferrihydrite was synthesized. Ferrihydrite is a poorly crystalline ferric oxide mineral that is more accessible to microbes than other more crystalline forms such as goethite and hematite (Cutting *et al.* 2009). It was synthesized using methods described in Straub *et al.* (2005). This methodology involves neutralizing a 0.4 M solution of  $\text{FeCl}_3$ , drop by drop with sodium hydroxide. To do this, the  $\text{FeCl}_3$  solution was added to a glass beaker on a stirring plate with a calibrated pH electrode placed in it. The pH was adjusted to 7 by adding 1 M NaOH one drop at a time with a burette. This formed a precipitate, which was left to settle for 24 hours and was then transferred to 250 ml centrifuge bottles and centrifuged at 12,000 rpm for 20 minutes (Sorvall RCB5 centrifuge with GSA rotor, Sorvall, UK). The supernatant was then removed, the pellet re-suspended in 200 ml of  $\text{dH}_2\text{O}$  and re-centrifuged. After each centrifugation, the conductivity was measured until it reached levels less than a 0.01 % NaCl solution. The solution was then centrifuged a final time and re-suspended at approximately a 1 M concentration in distilled water. The concentration was checked using the ferrozine assay as described below (see Section 7.1.1.1) with an additional step. This involved taking five 100  $\mu\text{l}$  sub-samples and acidifying them with 4.9 ml of 0.5 M HCl and 200  $\mu\text{l}$  of 6.25 M hydroxylamine-hydrochloride to reduced all the ferric iron to ferrous iron. These were then diluted 100x in  $\text{dH}_2\text{O}$  and 50  $\mu\text{l}$  transferred into the 2.45 ml of ferrozine solution and the concentration determined as described in 7.1.1.1. The resulting 1 M ferrihydrite was stored at 4 °C.

Before adding to the media, a sterile stock solution of ferrihydrite was created by moving 80 ml of the 1 M solution into a serum bottle, crimp sealing it with a rubber bung and aluminum top, sparging it with  $\text{N}_2$  for 10 minutes and then autoclaving it. This stock was kept for a maximum of 6 months to prevent the formation of crystalline goethite and hematite (Straub *et al.* 2005).

The basal media was made up in the same way as described in Nixon and Cockell (2015), with the addition of NaCl at 5 different concentrations (see Table 7.1). These ingredients were mixed together in a larger glass beaker and added to 200 ml serum bottles (50 ml), crimp sealed with a rubber stopper and aluminum top, sparged with N<sub>2</sub>/CO<sub>2</sub> (80:20) for 10 minutes and autoclaved. The enrichments were then amended with sterile anaerobic stocks of acetate and lactate (5 mM each) as electron donors and ferrihydrite (50 mM). Finally, FeCl<sub>2</sub> was added at a concentration of 1.3 mM as a reducing agent.

Chemical	Quantity
NaCl	250.0, 150.0, 50.0, 10.0 g/l
NaHCO <sub>3</sub>	2.5 g/l
KCl	0.2 g/l
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.06 g/l
NH <sub>4</sub> Cl	0.25 g/l
Vitamin solution (141)	10 ml
Trace elements (320)	10 ml
Top up with dH <sub>2</sub> O	1000.0 ml

**Table 7.1.** Basal Fe(III) reducer media recipe. NaCl concentrations varied between the different enrichments. The vitamin solution contained 2.0 mg/l biotin, 2.0 mg/l folic acid, 10.0 mg/l pyridoxine-HCl, 5.0 mg/l thiamine-HCl·2H<sub>2</sub>O, 5.0 mg/l riboflavin, 5.0 mg/l nicotinic acid, 5.0 mg/l D-Ca-pantothenate, 5.0 mg/l, 0.1 mg/l vitamin B12, 5.0 mg/l p-aminobenzoic acid and 5.0 mg/l lipoic acid. The trace element solution contained (in g/l dH<sub>2</sub>O): MnSO<sub>4</sub>·H<sub>2</sub>O (0.5), NaCl (1.0), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.1), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.1), CoCl<sub>2</sub>·6H<sub>2</sub>O (0.1), MgSO<sub>4</sub> (3.0), ZnCl<sub>2</sub> (0.13), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.01), nitrilotriacetic acid (1.5), AlK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O (0.01), H<sub>3</sub>BO<sub>3</sub> (0.01), NaMoO<sub>4</sub> (0.025), NiCl<sub>2</sub>·6H<sub>2</sub>O (0.024) and NaWO<sub>4</sub>·2H<sub>2</sub>O (0.025). Vitamin and trace element mixes were stored at 4°C in the dark. pH was balanced to 7.5 using N<sub>2</sub> and N<sub>2</sub>/CO<sub>2</sub> gas mixtures.

Once the media was complete in the serum bottles, 500 µl of the anaerobically collected brine samples from the mine were added to each. Two brines, 215 and Billingham, were used to start the enrichments. The enrichment cultures were then incubated at 37 °C in the dark and the levels of Fe(II) production measured.

### 7.1.1.1 Determining Fe(II) concentration

As is typical in the literature (e.g. Nixon and Cockell 2015), the production of Fe(II) was used as a proxy for microbial growth through iron reduction in the cultures. This was measured by using a ferrozine assay (Stookey 1970). To achieve this, 100 µl of sample was removed from each enrichment culture and added to 4.9 ml of 0.5 M HCl using a sterile syringe and needle and left to digest for an hour. From this digestion, 50 µl was removed and added to a cuvette containing 2.45 ml of ferrozine solution, mixed with a pipette tip and measured on a spectrophotometer (Helios alpha, Thermo Fisher Scientific, Waltham, MA, USA) at 562 nm.

The ferrozine solution was made up of 11.96 g/l HEPES and 1 g/l ferrozine [5,6-Diphenyl-3-(2-pyridyl)-1,2,4-triazin-4,4'-disulfonic acid disodium salt hydrate] in dH<sub>2</sub>O and pH corrected to 7. The solution was stored in the dark at 4 °C for a maximum of 4 weeks.

Standards used to calibrate the measurements were 50 mM (0.139 g FeSO<sub>4</sub>·7H<sub>2</sub>O in 10 ml 0.5 M HCl), 20 mM, 10 mM, 5 mM and 1 mM. Each was freshly made for every batch of ferrozine to ensure measurement calibration remained accurate. Ferrous iron concentration was determined through using a linear regression equation from the calibration graph generated by measuring these standards. Cultures were measured every 20-30 days.

### 7.1.2 Enrichment of sulphate reducers

A modified DSMZ 1055 recipe was used as the base enrichment media (Table 7.2) for the sulphate reducers. Additional NaCl was added to the media to better reflect the conditions in the brines. The resazurin was removed since it was found to cause issues with the diamine assay (see Section 7.1.2.1). Unlike the iron reducer enrichments, the sulphate reducers were only enriched at 250 g/l NaCl, since the iron reducer media was most successful at this concentration (see Section 7.2).

Solution	Chemical	Quantity
Part 1	NaCl	250.0 g
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	10.0 g
	KCl	6.0 g
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.4 g
	NH <sub>4</sub> Cl	1.0 g
	KH <sub>2</sub> PO <sub>4</sub>	0.1 g
	Yeast extract	0.5 g
	Trace elements (320)	1.0 ml
	Selenite and tungstate (385)	1.0 ml
	Vitamin solution (141)	10.0 ml
	Top up with dH <sub>2</sub> O	850.0 ml
Part 2 (add 50 ml to part 1)	NaHCO <sub>3</sub>	80.0 g/l
Part 3 (add 50 ml to part 1)	Sodium lactate	50.0 g/l
Part 4 (add 50 ml to part 1)	Na <sub>2</sub> S·9H <sub>2</sub> O	6.0 g/l

**Table 7.2.** Modified DSMZ recipe used to enrich sulphate reducing organisms. Once all the parts were mixed together the final volume was 1 liter. The vitamin solution was the same used for the iron reducer media (see Table 7.1). Trace element solution (SL-10) contained 10 ml of 7.7 M HCl, 1.5 g/l FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.07 g/l ZnCl<sub>2</sub>, 0.1 g/l MnCl<sub>2</sub>·4H<sub>2</sub>O, 6.0 mg/l H<sub>3</sub>BO<sub>3</sub>, 0.19 g/l CoCl<sub>2</sub>·6H<sub>2</sub>O, 2.0 mg/l CuCl<sub>2</sub>·2H<sub>2</sub>O, 24.0 mg/l NiCl<sub>2</sub>·6H<sub>2</sub>O, 36.0 mg/l Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O and 0.5 g/l Na<sub>2</sub>-EDTA and the pH adjusted to 7.5. The selenite-tungsten solution was made up of 3.0 mg/l Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O, 0.5 g/l NaOH and 4.0 mg/l Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O. pH was balanced to 7.5.

This base media was mixed and added to 200 ml serum bottles in volumes of 42.5 ml, sparged with N<sub>2</sub> and autoclaved. Parts 2 and 3 were mixed in serum bottles, sparged at the gas station and autoclaved. Part 4 was mixed in an anaerobic chamber to prevent the Na<sub>2</sub>S·9H<sub>2</sub>O oxidising, using dH<sub>2</sub>O that had been sparged with N<sub>2</sub>. The bottles were then sealed and autoclaved.



Once all the parts had cooled, 2.5 ml of parts 2, 3 and 4 were added to the serum bottles containing part 1 using sterile anaerobic techniques at the gas station (see Chapter 4). The pH was then checked and balanced as required. Inoculums of 500 µl of anaerobically collected brine were then injected into the media. Again, two brines, 215 and Billingham were used to start the enrichments. Enrichments were carried out in triplicate at 37 °C in the dark.

#### **7.1.2.1 Determining S<sup>2-</sup> concentration**

The production of sulphide was used as a proxy for microbial sulphate reduction. Here, an assay based on the Cline (1969) method with adaptations from Reese (2011), was used to monitor sulphide concentrations in the cultures during incubation. Firstly, a standard curve was generated to calibrate the spectrophotometer readings with sulphide concentrations. A variety of standards were made up, covering an estimated range of sulphide concentrations expected to be produced by the enrichments should sulphate reducers be present. These were created in the same way as part 4 of the media, by solving Na<sub>2</sub>S.9H<sub>2</sub>O in dH<sub>2</sub>O inside the anaerobic chamber in serum bottles. These were then crimp sealed with rubber bungs and aluminum tops. The concentrations used for the standards were 0.75, 1.25, 2.5, 3.75, 6.25 and 10 mM.

The standards and enrichment cultures were measured in the same way. One hundred microliters of each was added to 4.9 ml zinc acetate solution. This was composed of zinc acetate (10 g/l) and dH<sub>2</sub>O up to 1 l. Two hundred microliters of the zinc acetate and standard/sample solution was then added to 800 µl of dH<sub>2</sub>O in a cuvette, mixed with a pipette tip, and 80 µl of diamine reagent added. The diamine reagent was made up of N,N-dimethyl-p-phenylenediamine sulphate (4 g/l), FeCl<sub>3</sub>.6H<sub>2</sub>O (6 g/l) added to 50 % (v/v) HCl in a glass bottle (stored at 4 °C in the dark). The mixture of diamine reagent, zinc acetate and sample was then left for 30 minutes to allow the reaction to develop. Finally, absorbance was measured at 670 nm on the spectrophotometer. This whole process was carried out in triplicate. A new standard curve was generated for every batch of diamine reagent. Cultures were measured every 20-30 days.

## 7.2 Results

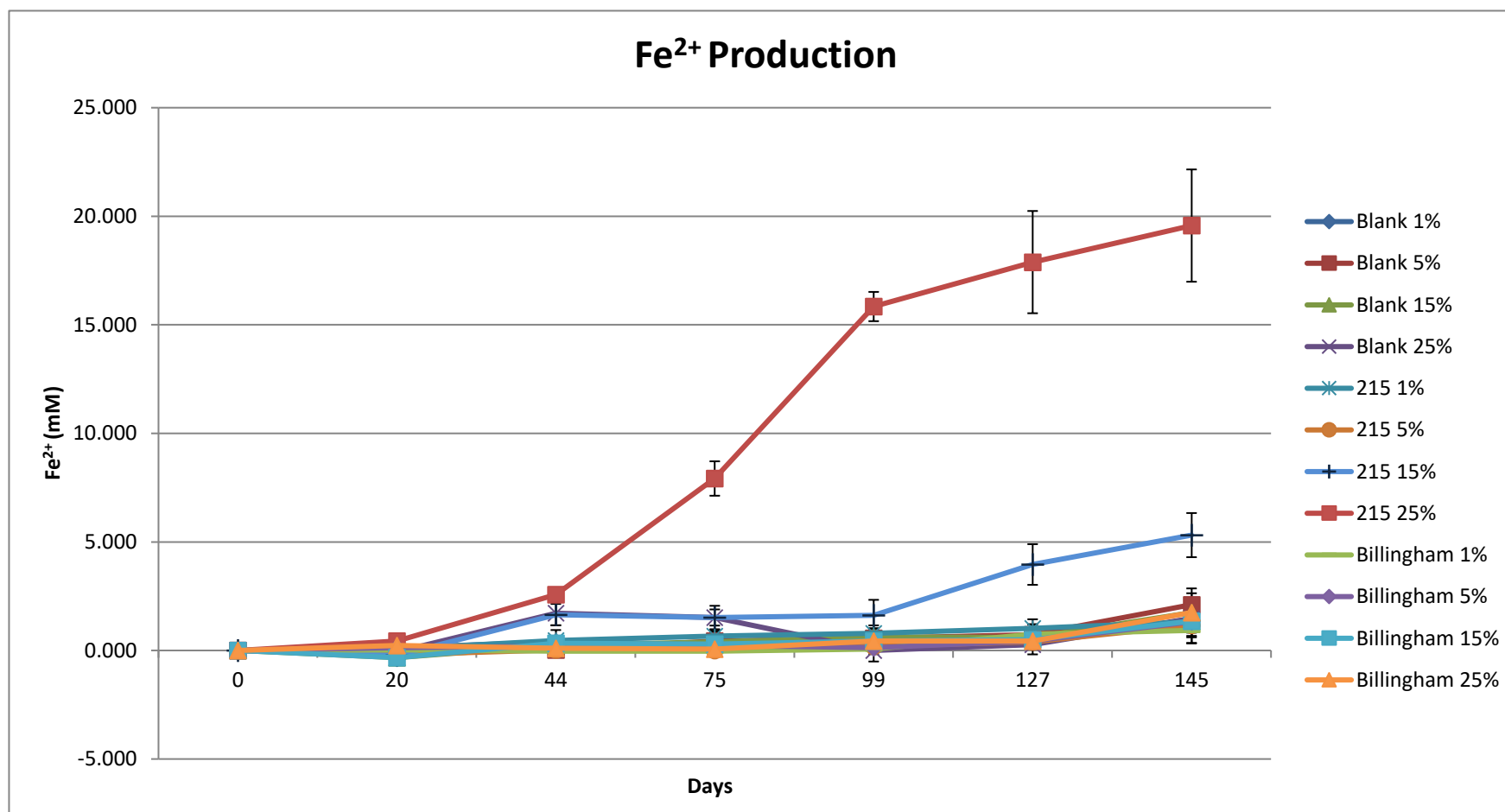
Both the iron and sulphate reducing cultures produced  $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  in a manner consistent with microbial production.

### 7.2.1 $\text{Fe}^{2+}$ production in enrichment cultures

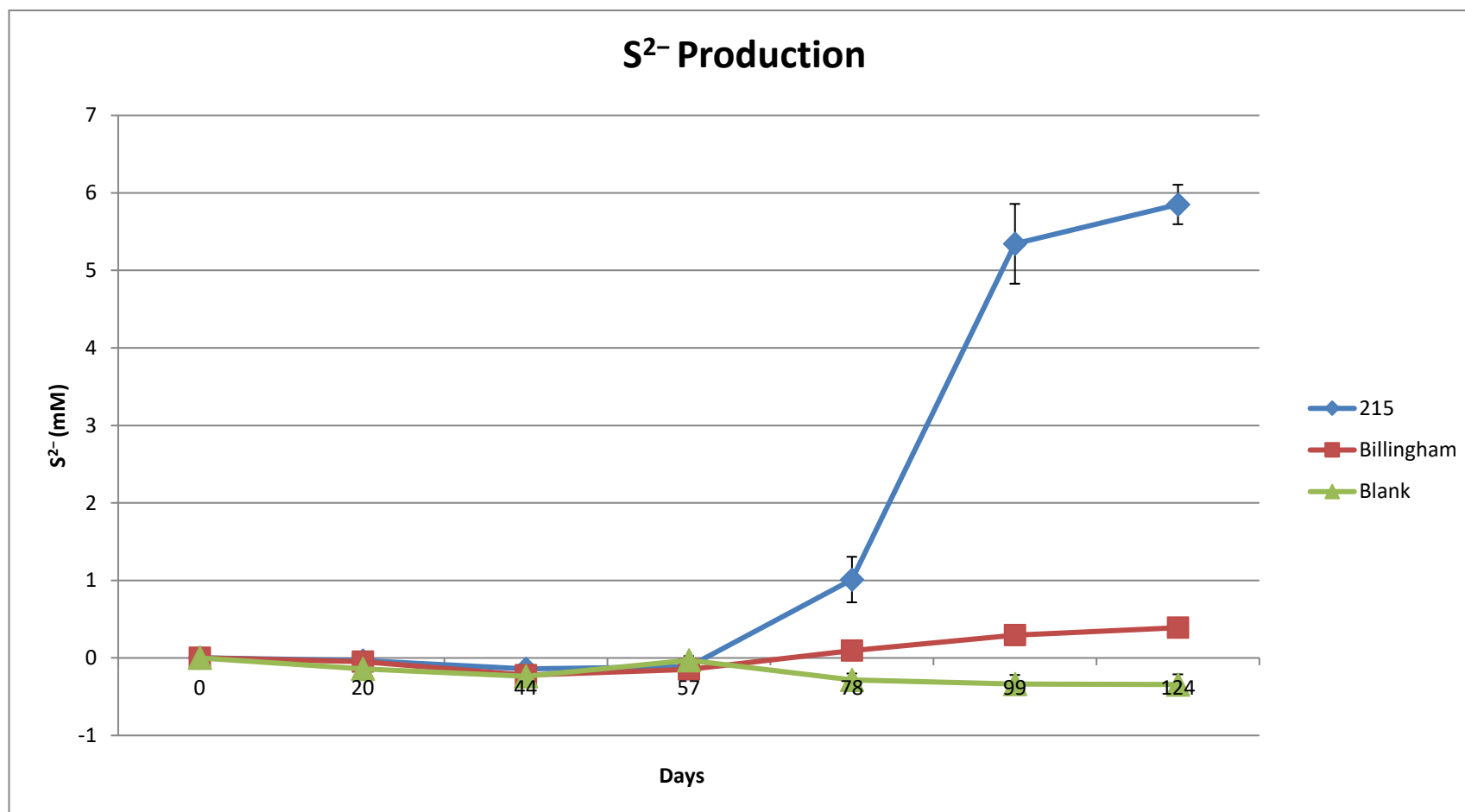
As discussed, iron reducing cultures were set up over a range of salinities.  $\text{Fe}^{2+}$  production was only detected in the 15 % and 25 % NaCl media inoculated brine 215, and not at the 5 % and 1 % of 215 or any of the enrichments inoculated with Billingham. This production was strongest at 25 % NaCl. In this enrichment,  $\text{Fe}^{2+}$  production began to move away from the blanks at the 44-day time point. At the end of the experiment, after 145 days, around 19.5 mM of  $\text{Fe}^{2+}$  had been produced by the 25 % NaCl culture in comparison to roughly 5.3 mM in the 15 % culture. This was against a baseline of around 1.3 to 2.1 mM produced in the 5 %, 1 % and blank media. Both 5 % and 1 % were within the errors of the blank media and so effectively did not show any evidence for  $\text{Fe}^{2+}$  production (see Figure 7.1).

### 7.2.2 $\text{S}^{2-}$ production in enrichment cultures

As mentioned sulphide production was only investigated at 250 g/l due to the results observed in the iron reducing cultures. Sulphide production was again strongest in the enrichments started with brine 215. Here, sulphide production was first observed at the 78-day time point, and reached around 6 mM by the end of 124 days. These levels were significantly higher than in the Billingham culture, which only displayed a modest increase to around 0.3 mM (Figure 7.2).



**Figure 7.1.** Fe<sup>2+</sup> production in media with varying NaCl concentrations over 145 days. Triplicate data points are experimental triplicates, i.e. three measurements were taken from each enrichment culture with the ferrozine assay and the average of those measurements used to represent one of the triplicate data points plotted on the graph. In this way, each time point is three individual cultures. The error bars therefore represent experimental triplicates.



**Figure 7.2.** Sulphide production over a period of 124 days in the sulphate reducer enrichment cultures. As in Figure 7.1, error bars represent experimental triplicates as in Figure 7.1.  $S^{2-}$  production was again significantly higher in the enrichment cultures inoculated from brine 215. Billingham displayed some low levels of  $S^{2-}$  production.

## 7.3 Discussion

### 7.3.1 The presence of microbial iron and sulphate reduction in a deep subsurface evaporite deposit

The pattern of  $\text{Fe}^{2+}$  production in the enrichments was consistent with microbial iron reduction. Distinct lag, exponential and stationary phases appear to be present in the production of  $\text{Fe}^{2+}$  in the 215 culture at 25 % NaCl. The 215 15 % NaCl enrichment does show an increase in  $\text{Fe}^{2+}$  over the blanks and other cultures of around 3–4 mM, but the distinct growth-like phases are less clear. The patterns of sulphide production in the 25 % media were similar. 215 displayed clear lag, exponential and stationary phases. Sulphide production patterns were less reminiscent of microbial growth curves in the Billingham culture, despite its small increase over the blank.

It is very likely that the ferrous iron or sulphide production observed is related to microbial processes given the patterns of this production and the temperatures at which it is occurring. However, sequencing the cultures would be ideal to confirm this. Both Billingham and 215 metagenomes appeared to contain proportionally similar *Geobacter* components. *Desulfovibrio* and *Desulfohalobium* were also detected in both brines, but were slightly less proportionately abundant in brine 215. However, neither brine contained complete pathways for dissimilatory sulphate reduction in the metagenomes (see Chapter 5). Other studies have found genes related to microbial iron and sulphate reduction in hypersaline (e.g. Emmerich et al. 2012, Kjeldsen et al. 2007) and deep subsurface environments (Sass and Cypionka 2004). The results here suggest that organisms capable of dissimilatory iron and sulphate reduction are most likely present in the brines, but that these organisms are only a minor part of the brine community.

The difference in reduction signatures in the enrichments started by each brine may be related to their exposures to oxygen. Billingham was a large brine pool that had been exposed to oxygen in the mine air for significantly longer than 215, which was sampled just a few meters down slope from where it exited the wall. The additional exposure to oxygen may have rendered any oxygen sensitive anaerobic organisms present in the brine inactive. Their genetic signatures would still be present in the brine, but they would be unable to function or may function at a greatly reduced rate.

One additional point worth highlighting is that some organisms have been shown to reduce ferric iron, but not to gain energy (Lovley 2013). Such processes may be incidental consequences of many dissimilatory sulphate reducers hosting cytochrome (c3), which can act as a ferric iron reductase (Lovley et al. 1993). Alternatively, these organisms could be trying to change their surrounding environment to improve conditions for sulphate reduction (Lovley 2013). The increases in the ferrous iron observed in the enrichments may therefore be unrelated to organisms reducing ferric iron to directly produce ATP.

The production of ferrous iron and sulphide are likely related. A number of dissimilatory sulphate reducing organisms have also been shown to be able to carry out iron reduction, usually once the sulphate supply has been exhausted, such as *Desulfosporosinus lacus* (Ramamoorthy et al. 2006), *Desulfuromonas acetoxidans* (Roden and Lovley 1993) or *Desulfofrigus oceanense* (Knoblauch et al. 1999). It is entirely possible that these types of organisms are responsible for both the sulphate and iron reduction observed.

### 7.3.2 Link to Martian habitability

Working with the hypothesis that the observed production in sulphide and ferrous iron were related to microbial processes allows speculation around the plausibility of these metabolisms to exist on Mars. As mentioned in Chapter 2, the deep subsurface of Mars will exhibit dark, anaerobic environments. In this context, redox metabolisms provide a strong candidate for metabolisms present in the Martian deep subsurface (Nixon et al. 2013), particularly those involving sulphate and ferric iron minerals which have been detected on the planet's surface (e.g. Christensen et al. 2001a, b, Klingelhöfer et al. 2004, Gendrin et al. 2005). Some of these environments are also likely to be hypersaline due to the abundance of evaporite minerals present. If the  $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  production observed is biologically produced, it would indicate that viable organisms capable of these metabolisms are present in terrestrial deep subsurface evaporite brines. This further strengthens the case that these are plausible metabolisms for survival in the Martian deep subsurface, demonstrating the ability of such organisms to cope with all the typical extremes of a deep subsurface environment, but with the additional stressor of high concentrations of NaCl.

## 7.4 Limitations and further work

Due to the incubator malfunction, a number of aspects could be added to this study to better test for the presence of these two metabolisms. Being unable to sequence the cultures was a key limitation on this work. This would therefore be the natural next step. After growing and monitoring a new set of enrichment cultures, and transferring them multiple times, DNA could be extracted and phylogenetically examined for organisms related to known dissimilatory iron and sulphate reducers. Additionally, assays for key marker genes responsible for these metabolisms could be carried out on the cultures. The combination of this additional work would more robustly test for the presence of these metabolisms.

The work could then be expanded significantly. If a novel new species is suspected, it could be isolated and characterised. Other metabolisms, such as perchlorate reduction, could be examined to see if the organisms in the brines are able to carry out other Mars relevant metabolisms. Any strains isolated from the enrichment cultures could be exposed to Martian conditions in a simulation chamber to examine their survival periods and residual biosignatures.

## 7.5 Conclusion

The work here was unfortunately limited due to the equipment malfunction experienced. However, it does give a foundation for future work to build on. A number of conclusions can be made from these experiments:

- $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  production are present in enrichment cultures inoculated by brine 215 in Boulby. The pattern of this production is consistent with production by microbial organisms. Organisms capable of iron and sulphate reduction are therefore hypothesised to be present in small numbers in some of the brines in Boulby mine.
- Ferric iron reduction was strongest at the highest salinity tested (250 g/l), indicating that the organisms responsible for this process function at extreme NaCl concentrations.

- Of the two brines investigated,  $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  production was only observed in brine 215. This could have been for a number of reasons, but greatly increased exposure to oxygen in Billingham which may have killed oxygen sensitive dissimilatory iron and sulphate reducers is the favoured hypothesis.

Further work on these metabolisms in a range of deep subsurface hypersaline environments will help determine how organisms survive and cycle elements in the terrestrial deep subsurface. This improved knowledge of the terrestrial deep subsurface, combined with a better understanding of the Martian subsurface through future missions will be important in helping to select the best locations to search for life on the red planet.



## Chapter 8: Concluding discussion

This final chapter summarises the main findings of each previous chapter and attempts to synthesise the results to update the knowledge gap and provide a direction for future workers.

### 8.1 Summary of main findings and synthesis

Each study in this thesis had its own set of conclusions, many of which informed and directed other studies. Chapter 3 primarily examined origin and evolution of the brines. This was to provide context to the results of later chapters and allow any links between the community present and the inferred origin and evolution of each brine to be teased out. These brines appear to have taken a variety of pathways through the deep subsurface, resulting in them interacting with a diversity of different evaporite minerals that imparted a range of ions to the brines. Through elemental and isotopic analysis, it was inferred that most brines (Billingham, 215, 44 XC and 29 XC) have their origin in the Sherwood Sandstone aquifer which sits above the mine. Billingham, 215 and 44 XC were Na/Cl dominated, whilst 29 XC was K/Cl dominated. Brine 101-P appeared to have a very different origin. These waters appear to have interacted with dolomite and became more concentrated in Mg than the other brines. Chapter 3 also identified a range of potentially biologically useful chemical species in the brines, including nitrate and sulphate, and several gases such as molecular hydrogen, methane, ethane, propane and n-butane, trapped in sylvinite rock. These gases appear to have a thermogenic origin and were most likely incorporated into the sylvinite through interactions with the large quantity of clay minerals contained in the salt rock.

The study detail in Chapter 4 measured a range of previously identified physicochemical measurements demonstrated to limit habitability in brines, including chaotropicity, water activity and ionic strength. These results were then combined with culturing experiments to determine the habitability of each brine. All brines were shown to be habitable, except brine 101-P. This appeared to be due to its raised Mg levels (three times higher than any other brine). Interestingly, Brine 29 XC appeared habitable from the physicochemical

measurements carried out, but consistently provided negative enrichment results, suggesting the brine either contained inactive/dead organisms, or no organisms at all. These data demonstrate that brine origins and migration pathways can have a severe impact on fluid habitability within a deep subsurface evaporite deposit. In this way, the Zechstein Sea still influences the habitability of the modern deep subsurface.

Chapter 5 then examined the DNA profile of three brines using metagenomic approaches. DNA extractions failed in brines 101-P and 29 XC, supporting the habitability the conclusions made in Chapter 4 about 101-P. It also suggested that other environmental extremes may have been responsible for the lack of organisms in 29 XC. Successful DNA extractions were achieved from brines 215, Billingham and 44 XC. Analysis of these metagenomes revealed communities functionally and taxonomically similar to surface near saturation brines. This suggests that in the majority of cases, the structure of communities present in saturated Na/Cl brines are almost exclusively controlled by these ions, rather than any other environmental difference between the surface and subsurface. This agrees with a number of previous studies which examined subsurface sediments from the Dead Sea (Thomas et al. 2014). Several subtle differences between the surface and subsurface were identified however. This included the increased presence of genes related to denitrification in the subsurface brines, which may suggest this metabolism is more important in the hypersaline deep subsurface in comparison to the surface. Fermentation appeared to be a viable metabolism in both.

Using brine communities from the mine as inoculum, enrichment work was carried out in Chapter 6 to determine if any carbon sources were present in ancient salt that might promote growth in the brine communities. Within the confines of the experimental design, no evidence was found that any carbon entrapped within the salt rocks could itself provide the organisms in the brines with a usable carbon source. A fertilisation effect was observed, however, when the salt rocks, particularly sylvinite, were added to the enrichment media. The brine communities were also shown to utilise a range of organic compounds for growth, including oil seeps collected from the mine, under both anaerobic and aerobic conditions. This range of organic compounds appears more restricted under anaerobic conditions and further work is required to determine which carbon sources present in the oil seeps were being utilised by the organisms for growth. Additionally, the ability of the brine communities to grow in mineral media with no added carbon source underlined the capacity of these halophiles to grow in oligotrophic environments.

In the final data chapter (7) of this thesis, attempts were made to enrich for organisms from the brines that use two highly Mars relevant metabolisms; dissimilatory sulphate and iron reduction. These attempts appear to have been successful, with  $\text{Fe}^{2+}$  and  $\text{S}^{2-}$  production resembling the patterns typically expected for microbial growth patterns. However, further analysis of the DNA in the enrichments is required to confirm the presence of these types of organism. The metagenomic work in Chapter 5 suggested that only very small quantities of the community were made up of organisms capable of dissimilatory sulphate and iron reduction. Pathways for dissimilatory sulphate reduction were also incomplete. Overall, the combination of results from these studies suggest that whilst both types of organism are present in the brines in small quantities, they likely have limited importance to the overall environment.

## 8.2 Implications

This work reveals for the first time the influence of salts on the changing diversity of organisms in the deep subsurface and how changes in fluid migration can make the difference between habitable and uninhabitable conditions. These results have several implications for both terrestrial and extraterrestrial deep subsurface environments.

The research demonstrated that organisms present in deep subsurface evaporite brines can metabolise a number of carbon compounds, potentially including hydrocarbons, in a dark anaerobic hypersaline environment. Despite no work being done to examine RNA, this suggests that these organisms are capable of activity in deep subsurface evaporites, given their tolerance to salinity, darkness and oligotrophic environments. The fertilisation effect, observed by artificially amending mineral media to geochemically mimic the solvings of natural salt minerals, particularly sylvinite, could also help encourage the activity of these organisms in the deep subsurface when carbon levels are low.

Hydrocarbon deposits could play an important role in slowly amending the Boulby field site with carbon sources over geological time. This includes both as thermogenic gases, as found trapped in the sylvinite clay minerals, and liquid hydrocarbon seeps. Hydrocarbon deposits are often found below Permian evaporates, meaning this process could be widespread. Whilst the metagenomes showed that genes involved in methanotrophic or hydrocarbon degrading pathways were absent, organisms capable of these metabolisms could still be

present in the brines in small numbers, or, particularly in the case of hydrocarbon degrading organisms, may be poorly characterised.

The metagenome provided little evidence of viable autotrophic metabolisms in the brines and the community is dominated by chemoorganotrophs. This continues to demonstrate that chemoheterotrophic metabolisms are very important in the terrestrial deep subsurface, particularly in hypersaline environments. On other planetary bodies like Mars, a lack of significant carbon input into the deep subsurface from millions of years of extensive carbon fixation by photosynthesis, could restrict deep subsurface habitability. This may be particularly true in any Na/Cl dominated brines like those found in Boulby, due to thermodynamic demands of living at high concentration of these ions and lack of light.

This relationship between the surface and subsurface is important to consider when speculating on potential biogeochemical cycles that might be occurring in evaporite sequences. From the data gathered during this thesis, there is little evidence of a full active biogeochemical cycle occurring over short time periods in the evaporite sequence. For example, no evidence was found from the metagenomic work that the habitable brine communities were capable of complete sulphur or nitrogen cycles, or could autotrophically fix carbon. Instead, the organisms appear to rely on products of photosynthesis and other biogeochemical cycles in adjacent environments, rather than maintaining their own complete biogeochemical cycles. Successful long-term community maintenance would be possible with this mode of life given the organisms ability to deal with long periods of dormancy, radiation and oligotrophic conditions (see Section 2.1.1.3).

This work did not examine the recurring question of long term microbial survival in deep subsurface evaporites. However, nothing covered in this thesis suggests these environments are isolated from the surface over geological time. Brine seeps were shown to have moved from overlying regions containing much younger waters. This constant input of material suggests that despite their isolated appearance, the deep subsurface evaporites at this site are linked to modern surface environments, which may help explain the modern appearance of the organisms trapped in the salt crystals (Graur and Pupko 2001). Through this constant delivery of nutrients over geological time, it is possible that halophiles have inhabited the sequence since it was deposited. However, communication to other subsurface and ultimately surface environments means that the community probably has not evolved independently of other groups of halophiles.

Denitrification appears to be an important metabolism in deep subsurface evaporite brine communities. Although only brine 44 XC had detectable nitrate levels, it may be that nitrate is more common in deep subsurface brines in comparison to surface hypersaline environments. Nitrate had previously been detected in Mars meteorites (Kounaves et al. 2014) and was discovered *in-situ* by the SAM instrument on the Curiosity rover in 2015. Given the lack of oxygen on Mars, this could be an extremely important compound in enhancing habitability in the planet's subsurface, as demonstrated by its apparent importance in their terrestrial counterparts.

Brine seeps found in close proximity were found to have drastically different habitability characteristics depending on their migration route through the deep subsurface. This highlights that habitability in subsurface brine environments can be extremely precarious. Brines in the deep subsurface of Mars or Europa could have very different compositions, but be equally susceptible to localised shifts in habitability, particularly if a very active hydrological cycle is present. Brine 29 XC also highlights a general need to consider multiple extremes when assessing habitability. Solely relying on established physicochemical parameters for determining brine habitability is inadequate.

## 8.3 Remaining knowledge gap and further work

Whilst this work has advanced our knowledge of deep subsurface evaporites in a number of areas, a range of knowledge gaps remain that future workers should address. These include:

- Determining if halophiles are active in these deposits would be a valuable contribution of the field. Probing these environments *in-situ* with RNA assays could be one way of approaching this problem. This could also be used as another tool to examine habitability in the most extreme brines.
- More work should be done to investigate the presence of brines with atypical compositions, particularly at the edge of habitability. This may uncover new organisms with novel adaptations able to proliferate in extremes not typically seen on Earth. For example, very little work exists pertaining to sulphate brines. These brines are typically uncommon on Earth and none have been observed in Boulby Mine. However, with the range of sulphate minerals making up important parts of most evaporite sequences, deep subsurface sulphate brines should be present in a range of places. The Basque lakes in Canada, for example, are thought to be sourced from the

subsurface. Sulphate brines are particularly Mars relevant due to extensive sulphate salt deposits making up a significant part of Martian stratigraphy.

- Further work is required to confirm the presence of dissimilatory iron and sulphate reducing organisms in these brines. Other relevant Mars redox couples, such as perchlorate reduction, could also be examined.
- Understanding hydrocarbon utilisation in halophiles needs to be advanced significantly. Not enough is known about the metabolic pathways utilised by this group of organisms to metabolise these compounds. With metagenomic approaches becoming increasingly important in probing environmental microbiology, a lack of well characterised functional genes in this group of organisms may lead to the importance of these metabolisms being under- or overestimated.
- The metagenomes sequenced here showed little evidence of organisms able to utilise the gases trapped in the salt rocks. However, direct enrichments on these gases may still reveal organisms able to achieve this. This would better determine the importance of the link between hydrocarbon deposits and halophilic communities in overlying evaporites.
- The tolerance of halophilic communities from deep subsurface or surface environments to multiple extremes such as temperature or pressure would be valuable to better constrain. It may be at some confluences of these extremes, high Na/Cl concentrations recede as the dominant influence on community structure. Alternatively, an environment may become uninhabitable even when individually no one quantified extreme should be able to achieve this.
- To better examine if deep subsurface halophiles have the capability to exist on Mars, their ability to utilise electron donors thought to exist on the planet could be tested under anaerobic, dark conditions. Future workers could specifically look at the compounds found in meteorites like carbonaceous chondrites for example. The next generation of Mars rovers will significantly advance the search for organics on the red planet, so new information could be used to inform such studies.
- It is important to note that what may be true of the brines sampled here, may not be true of other deep subsurface evaporites. A continued increase in general characterisation data of these environments, including more next generation sequencing, would allow observations made at individual sites to be understood as general patterns.

## 8.4 Conclusion

The main objective of this study was to elucidate the role of salts in shaping the deep subsurface biosphere. In particular, the study focused on understanding the influences of brine environment and migration routes on fluid habitability, functional and taxonomic community structure and energy and carbon acquisition in deep subsurface evaporite brines. The data presented in the preceding chapters makes several advances in these areas. Habitability is shown to be a dynamic and variable phenomenon in deep subsurface brines, able to change over small spatial scales. Brine origin and migration route through the deep subsurface have potentially huge implications for this habitability. However, in Na/Cl brines without exceptionally high quantities of Mg, these migration routes appear to make little difference to the functional and taxonomic structure of the microbial communities. The same is apparent when comparing these deep subsurface brines to their surface counterparts. Organisms in these environments appear capable of actively utilising a variety of carbon compounds *in-situ* and acquiring energy with processes like denitrification. Overall this work shows deep subsurface evaporite deposits like Boulby to be complex and intriguing environments, that can teach us much about the habitability of other worlds.

# Appendix

Data tables are available through the The University of Edinburgh PURE system. Appendix F is available publicly on <https://www.mg-rast.org/>.

**Appendix A:** XRD Data

**Appendix B:** Trapped Gas Data

**Appendix C:** Chaotropicity and Kosmotropicity Assays

**Appendix D:** Ancient Carbon Growth Curves

**Appendix E:** Iron and Sulphate Reducer Growth Curves and Standards

**Appendix F:** MG-RAST Metagenome ID's 4678909.3, 4678908.3, 4705070.3



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